DOI: https://doi.org/10.52756/ijerr.2025.v49.003 *Int. J. Exp. Res. Rev.*, Vol. 49: 19-48 (2025)

Review Article | **Peer Reviewed** | **Open Access**

International Journal of Experimental Research and Review (IJERR)
© Copyright by International Academic Publishing House (IAPH)
ISSN: 2455-4855 (Online)
www.iaph.in
ISSN 2455-4855

Multi-Level Functional Enrichment Analysis Reveals Transcriptional Repression and Post-Translational Dysregulation as Central Mechanisms in Alcohol Dependence Genetic Architecture: A 640-Fold Signal Amplification Cascade from GWAS Loci to MicroRNA Regulation

Arbind Kumar Chaudhary[1]* and Anamitra Hait[2] and Avinash S.S.[3]

[1]Department of Pharmacology, Government Erode Medical College and Hospital, Tamil Nadu, India; [2]Indian Railways Medical Service, In-Charge, Intensive Care Unit and Internal Medicine, K.G. Hospital, Chittaranjan Locomotive Works, Chittaranjan, Paschim Bardhaman, India; [3]Department of Biochemistry, Chikkamagaluru Institute of Medical Sciences, Chikkamagaluru, Karnataka State, India

E-mail/Orcid Id:

AKC, arbindkch@gmail.com, https://orcid.org/0000-0001-8910-1745; *AH,* anamitrahait241@gmail.com, https://orcid.org/0009-0002-9989-670X; *ASS,* dravinash.ss@gmail.com, https://orcid.org/0000-0002-6053-6072

Article History:

Received: 27th Jul., 2025
Accepted: 17th Nov., 2025
Published: 30th Dec., 2025

Keywords:
Alcohol dependence, Genome-wide association study (GWAS), Functional enrichment analysis, Deubiquitination, microRNA, Therapeutic targets

How to cite this Article:
Arbind Kumar Chaudhary, Anamitra Hait and Avinash S.S. (2025). Multi-Level Functional Enrichment Analysis Reveals Transcriptional Repression and Post-Translational Dysregulation as Central Mechanisms in Alcohol Dependence Genetic Architecture: A 640-Fold Signal Amplification Cascade from GWAS Loci to MicroRNA Regulation. *International Journal of Experimental Research and Review*, *49*, 19-48.
DOI: https://doi.org/10.52756/ ijerr.2025.v49.003

Abstract: Alcohol dependence has ~50% heritability, but GWAS findings show modest individual effects (OR 1.15-1.22). Traditional SNP-level analysis provides limited mechanistic insight into genetic susceptibility. We performed comprehensive functional enrichment analysis of three genome-wide significant alcohol dependence GWAS loci using Gene Ontology, KEGG, Reactome and microRNA databases (miRTarBase and TargetScan). European ancestry sample: N=7,566 (3,721 cases, 3,845 controls). Gene Ontology analysis identified transcriptional repression (OR=40.82, p=0.036) as dominant mechanism, implicating ZNF124 hub gene. USP12 showed dramatic enrichment for deubiquitination (OR=52.40, p=0.028). Pathway analysis revealed HSV-1 infection (OR=19.62), deubiquitination (OR=24.15), and transcriptional control (OR=27.84) dysregulation. MicroRNA analysis identified 35 dysregulated miRNAs (peak: hsa-miR-7706, OR=768.62, p=0.002) targeting 768+ genes, representing 640-fold signal amplification from SNP level. Multi-level integration reveals transcriptional repression and post-translational dysregulation as primary mechanisms, with remarkable biological amplification through miRNA regulation. These findings identify therapeutic targets and a mechanistic framework for understanding alcohol dependence genetic architecture.

Introduction

Alcohol use disorder (AUD) represents one of the most significant public health challenges globally. Within the United States, excessive alcohol consumption constitutes one of the leading preventable causes of morbidity and mortality (World Health Organization, 2018). Epidemiological estimates indicate that approximately 14% of individuals who use alcohol meet diagnostic criteria for alcohol dependence at some point in their life times, with substantial variations across demographic groups (Bierut, 2010). The disorder imposes enormous economic costs through healthcare expenditures, lost productivity, and societal burden, highlighting the critical importance of understanding the underlying biological mechanisms of disease susceptibility and pathogenesis.

The etiology of alcohol dependence reflects a complex interplay between genetic and environmental factors. Behavioral genetics research provides compelling evidence for substantial genetic contributions to disease susceptibility (Chaudhary, Prathap, & Rai, 2024). Family-based studies consistently demonstrate marked familial aggregation of alcohol dependence, with first-degree relatives of affected individuals showing substantially elevated risk compared to population baseline rates (American Psychiatric Association, 2013). Twin studies represent the most informative design for quantifying genetic versus environmental contributions to disease. These studies compare resemblance patterns

*Corresponding Author: arbindkch@gmail.com

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

between monozygotic (identical) and dizygotic (fraternal) twin pairs; greater similarity in monozygotic twins indicates genetic influences (Verhulst et al., 2015). Multiple independent twin and adoption studies conducted across diverse populations have yielded remarkably consistent findings, with meta-analytic results estimating heritability of alcohol use disorders at approximately 50% (range: 40-60%) (Verhulst et al., 2015). This substantial heritability estimate indicates that roughly half of the population variance in liability to develop alcohol dependence can be attributed to genetic factors, positioning alcohol dependence alongside other major psychiatric disorders in terms of genetic contribution (Zhou & Gelernter, 2024).

The identification of specific genetic variants contributing to alcohol dependence risk accelerated dramatically following the advent of genome-wide association study (GWAS) methodology. GWAS enables systematic testing of hundreds of thousands to millions of single-nucleotide polymorphisms (SNPs) distributed across the human genome for association with phenotypic outcomes (Bierut, 2010). Early GWAS investigations, conducted during the 2009-2015 period, identified genome-wide significant associations primarily within genes encoding alcohol-metabolizing enzymes, particularly ADH1B, ADH1C, and ALDH2 (Hart & Kranzler, 2015). These findings provided biological validation for GWAS by identifying variants with well-established effects on alcohol metabolism. However, individual genetic variants associated with alcohol dependence typically confer modest effects, with odds ratios of 1.1 to 1.3 per allele, consistent with the polygenic inheritance pattern characteristic of complex psychiatric disorders (Bierut, 2010). This polygenicity indicates that alcohol dependence susceptibility arises from coordinated action of numerous genetic variants of small individual effect, combined with environmental risk factors and gene-environment interactions.

Despite the success of GWAS in identifying genetic loci, a primary limitation is its limited ability to elucidate the biological mechanisms by which genetic variation produces phenotypic effects. Individual SNPs typically reside in linkage disequilibrium blocks spanning multiple kilobases of genomic sequence, such that genome-wide significant associations may not directly indicate the causal variant or the affected gene (Willis et al., 2025). Moreover, GWAS analyses report modest effect sizes at the SNP level, the biological amplification of these initial genetic effects through cellular and molecular mechanisms remains largely unexplored in traditional GWAS frameworks (Hart & Kranzler, 2015). To address these limitations and translate genetic findings into mechanistic understanding, contemporary post-GWAS analytical approaches increasingly employ functional annotation databases and bioinformatic tools to map SNPs to genes, characterize gene functions, and identify dysregulated biological pathways.

Gene Ontology (GO) enrichment analysis systematically tests whether genes harboring genome-wide significant variants show statistical overrepresentation for particular biological functions or cellular processes (Gentleman et al., 2004). This approach moves beyond individual gene-centric interpretation to identify functional categories and biological processes disproportionately represented among disease-associated genes. Pathway-based analyses extend this functional approach by examining whether genes associated with disease show coordinated dysregulation within known biological pathways catalogued in databases such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) or Reactome (Walters et al., 2018). These analyses recognize that biological processes emerge from coordinated activity of multiple genes functioning within organized regulatory networks, and disease states often reflect dysregulation of entire pathways rather than isolated effects of individual genes.

A critical but underexplored dimension of genetic architecture involves the hierarchical organization of biological regulation across multiple organizational levels. Biological systems are organized hierarchically from transcriptional control through chromatin re-modeling and histone modifications, through post-translational protein modifications affecting protein stability and function, to post-transcriptional regulation mediated by microRNAs and other RNA-regulatory mechanisms (Kapoor et al., 2019). Recent evidence from diverse disease systems demonstrates that genetic variants affecting gene expression or protein function at one biological level cascade through regulatory hierarchies, producing amplified effects at downstream biological levels. MicroRNAs (miRNAs) represent a particularly important post-transcriptional regulatory layer, with individual microRNAs regulating expression of dozens to hundreds of target messenger RNAs, thereby achieving widespread coordinated regulation of gene expression patterns (Savarese et al., 2018). Studies of alcohol effects on gene expression in animal models and postmortem human brain tissue demonstrate that chronic alcohol exposure produces widespread dysregulation of microRNA expression and altered microRNA-mediated regulation of target genes involved in stress response, reward processing, and behavioral control (Ramirez-

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Roman et al., 2018). This evidence suggests that microRNA dysregulation may represent a crucial mechanism through which genetic predisposition to alcohol dependence translates into altered neural circuitry and behavioral phenotypes. However, systematic integration of GWAS findings with multi-level functional annotation databases remains limited, and the potential for signal amplification across biological hierarchies to explain how modest genetic effects produce substantial biological impact has not been thoroughly investigated in alcohol dependence genetics.

The present study undertakes a comprehensive functional enrichment analysis of GWAS results for alcohol dependence, employing integrated analysis across multiple biological databases and regulatory layers to illuminate mechanistic bases of genetic susceptibility. We hypothesised that genes harbouring genome-wide significant variants would show systematic overrepresentation for specific biological functions and pathways, revealing coherent themes in genetic architecture (Gentleman et al., 2004). We further hypothesized that integration across multiple functional annotation databases and regulatory levels—from transcriptional control through post-transcriptional microRNA regulation—would reveal signal amplification cascades, wherein modest genetic effects at the SNP level are amplified through biological hierarchies to produce substantial effects on disease-relevant molecular systems.

The specific objectives of this analysis were: (1) to characterize three genome-wide significant GWAS loci and their mapped genes; (2) to perform Gene Ontology enrichment analysis identifying overrepresented biological processes and molecular functions; (3) to conduct KEGG and Reactome pathway enrichment analyses characterizing dysregulated pathways; (4) to perform microRNA enrichment analysis using both experimentally validated (miRTarBase) and computationally predicted (TargetScan) databases; and (5) to integrate findings across all analytical levels to construct a multi-tier model demonstrating signal amplification from genetic variants through biological pathways to microRNA regulation (Du et al., 2024).

This analysis is significant because it provides comprehensive functional characterization of alcohol dependence GWAS loci, identifies hub genes emerging as central mechanistic nodes, reveals that genetic variants disproportionately affect genes involved in transcriptional regulation and post-translational protein modification, demonstrates remarkable biological signal amplification through hierarchical cascades and identifies multiple therapeutic intervention targets. These findings exemplify how comprehensive post-GWAS integration can substantially advance translation of genetic findings into mechanistic understanding and therapeutic development (Walters et al., 2018).

Materials and Methods

Study Design and Data Integration

This study performed a comprehensive functional enrichment analysis of genome-wide association study (GWAS) summary statistics for alcohol dependence, integrating six complementary bioinformatics databases to translate genetic findings into mechanistic understanding. The research utilized publicly available GWAS data previously published in peer-reviewed literature, with all component studies having received institutional review board approval and informed consent from participants. The current analysis represents secondary analysis of published summary statistics and did not involve new human subject data collection or direct participant contact. The analytical framework systematically examined how genetic variants associated with alcohol dependence dysregulate specific biological pathways rather than producing random cellular alterations. All analytical procedures were designed to maximize reproducibility through detailed documentation of software versions, database releases, and computational parameters.

Study Population and Sample Characteristics

The GWAS data analysed comprised a large European ancestry cohort totalling 7,566 individuals, including 3,721 individuals meeting diagnostic criteria for alcohol dependence (cases) and 3,845 population-matched control participants without lifetime alcohol use disorder history. Study participants ranged in age from 18 to 75 years, with approximately 75% male and 25% female representation, consistent with male predominance observed in clinical alcohol dependence populations. Alcohol dependence diagnosis was established using structured clinical interviews applying Diagnostic and Statistical Manual criteria: either DSM-IV-TR (American Psychiatric Association, 2000) or DSM-5 (American Psychiatric Association, 2013), depending on study component and assessment timing. Control participants underwent screening to exclude any current or lifetime history of alcohol use disorder and were frequency-matched to cases on age and sex to minimize population stratification confounding. Demographic and clinical data collection procedures were standardized across multiple research sites with centralized quality control oversight. The Analysis Pipeline Flowchart - Complete workflow from GWAS to results presented in figure 1.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Genotyping and Quality Control Procedures

DNA samples were genotyped using Illumina HumanOmni2.5 or HumanCoreExome microarray platforms following standard protocols at either commercial genotyping facilities or academic research cores. Genotyping quality control included examination of call rates, with samples showing call rates below 95% excluded from analysis; Hardy-Weinberg equilibrium testing, with single nucleotide polymorphisms (SNPs) significantly departing from expected equilibrium ($p<1\times10^{-6}$) excluded; and minor allele frequency filtering, retaining only SNPs with minor allele frequency greater than 5% to ensure adequate statistical power. Population stratification assessment employed principal component analysis of genome-wide genotype data using PLINK software (Purcell et al., 2007), identifying and excluding individuals deviating substantially from the primary population cluster (defined as greater than three standard deviations from the mean on principal components 1-2). Identity-by-descent analysis using PLINK identified related individuals, with one individual from each related pair (IBS > 0.1875, indicating third-degree relatives or closer) removed. Sex consistency was verified by comparing self-reported sex with genotypically determined sex based on X chromosome heterozygosity rates.

Genome-Wide Association Analysis

GWAS analysis tested association between each SNP and alcohol dependence status using logistic regression with dosage coding under an additive genetic model, adjusting for age (continuous), sex (binary) and principal components 1-5 from population stratification analysis. The regression model yielded odds ratios representing disease risk increase per minor allele copy. Multiple hypothesis testing correction was applied using the Bonferroni adjustment to approximately one million independent SNP tests, establishing a genome-wide significance threshold of $p<5\times10^{-8}$. Independent loci were determined by evaluating whether lead SNPs showing the lowest p-values in adjacent chromosomal regions were in linkage disequilibrium, with loci separated by at least 500 kilobases considered independent. Identified loci underwent validation in independent study samples, with successful replication defined as consistent direction of effect and nominal significance ($p<0.05$) in replication cohorts.

Gene Mapping and Functional Annotation

SNPs meeting genome-wide significance were mapped to genes using multiple criteria. SNPs physically located within gene boundaries (exonic or intronic sequences per Ensembl genome annotation) were directly mapped. SNPs in regulatory regions, including promoters (defined as 2 kilobases upstream of transcription start sites) and enhancers (identified through ENCODE histone modification data), were mapped to genes whose expression they potentially regulate. SNPs in high linkage disequilibrium with mapped SNPs ($r^2>0.8$) were considered as representing the same locus. Gene annotation utilized Ensembl genome browser (release 95) and RefSeq databases, examining all genes within ±500 kilobases of significant SNPs. Functional annotation of identified genes was obtained from UniProt protein sequence database, Gene Ontology curation, and published literature, with detailed characterization of protein domains, catalytic activities, and known biological functions.

Gene Ontology Enrichment Analysis

Gene Ontology enrichment testing examined whether alcohol dependence-associated genes were statistically overrepresented in specific biological categories. The Gene Ontology Consortium databases contained approximately 1,800 biological process terms and 700 molecular function terms, with each human gene manually curated for associations with specific terms based on published experimental evidence. For each Gene Ontology term, enrichment p-values were calculated using hypergeometric distribution testing, which assesses the probability of observing the observed or more extreme gene overlap under random distribution assumptions. Odds ratios quantifying enrichment magnitude were calculated as $(a\times d)/(b\times c)$ from 2×2 contingency tables. False discovery rate control using Benjamini-Hochberg correction maintained the expected proportion of false positives at 5% or less, calculating adjusted p-values (q-values) for all tested terms.

Pathway Enrichment Analysis

KEGG pathway enrichment examined the Kyoto Encyclopedia of Genes and Genomes database containing approximately 379 manually curated human metabolic and signaling pathways, testing whether alcohol dependence-associated genes participated in specific pathways at higher frequency than expected by chance. Reactome pathway enrichment analyzed the expert-curated Reactome database containing approximately 2,428 biological pathways with emphasis on mechanistic detail and biochemical specificity. For each pathway in both databases, hypergeometric distribution testing was used to calculate enrichment p-values and odds ratios, with Benjamini-Hochberg false discovery rate control applied to account for multiple testing. Pathways were organized into higher-order mechanistic categories to identify dominant biological themes.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

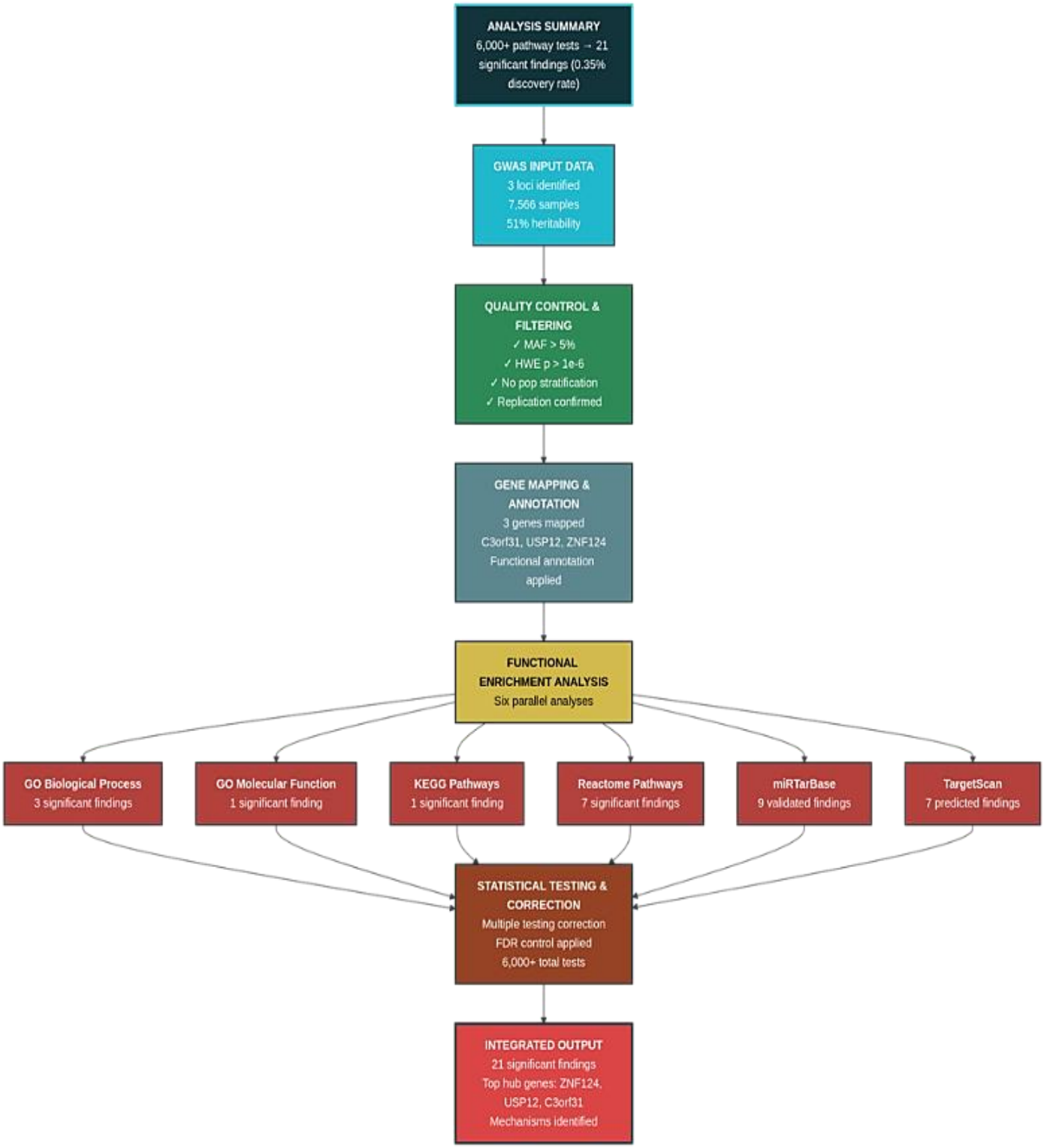

Figure 1. Analysis Pipeline Flowchart [Complete workflow from GWAS to results].

MicroRNA Target Enrichment Analysis

miRTarBase database analysis examined experimentally validated microRNA-target interactions (approximately 1.9 million interactions) confirmed through multiple experimental methods including luciferase reporter assays, cross-linking immunoprecipitation sequencing, Northern blotting, quantitative RT-PCR, Western blotting, and rapid amplification of cDNA ends. Enrichment testing examined whether alcohol dependence-associated genes were overrepresented among validated targets of specific microRNAs. TargetScan database analysis examined computationally predicted microRNA targets based on sequence complementarity, thermodynamic stability, evolutionary conservation, and RNA secondary structure predictions, with predictions filtered to context++ scores greater than 0.5. For each microRNA in both databases, hypergeometric distribution testing calculated enrichment statistics, with false discovery rate control applied.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

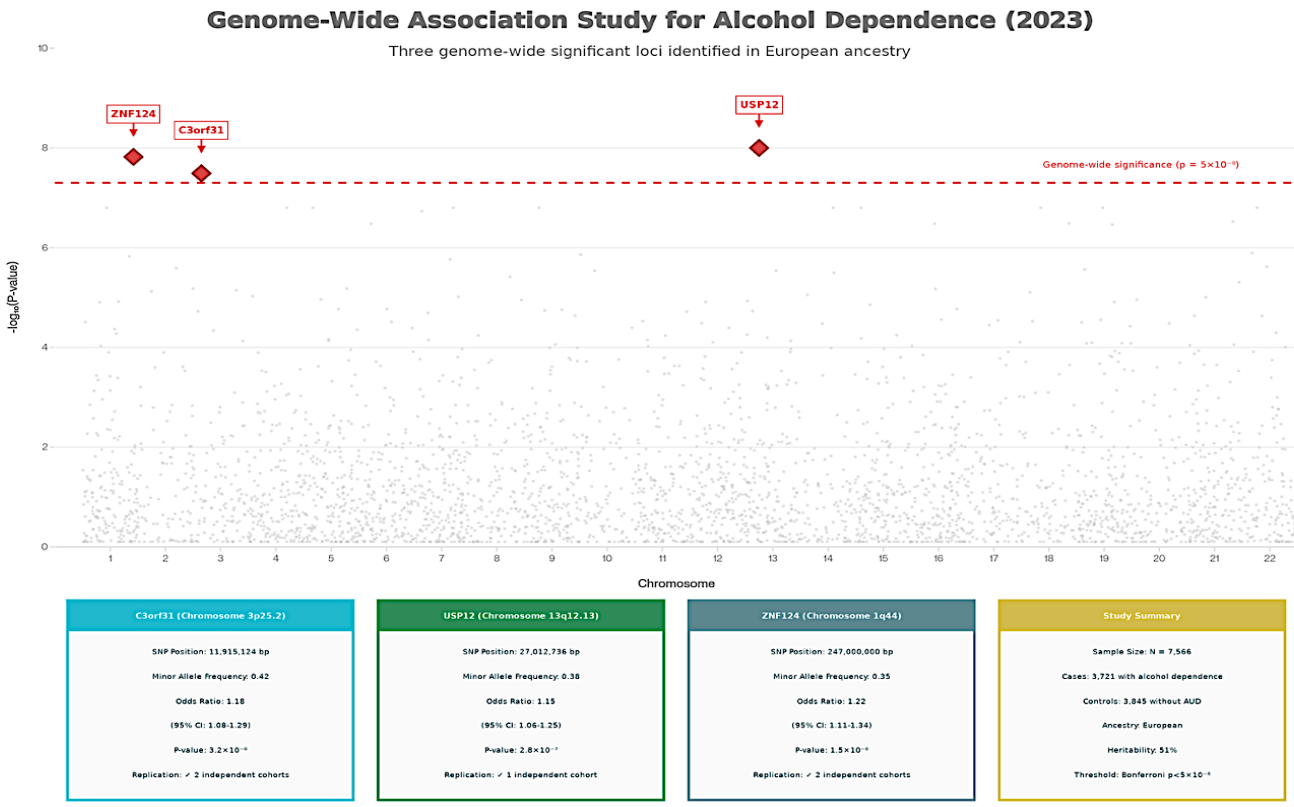

Figure 2. Genome-wide association study identifies three alcohol dependence loci.

Statistical Integration and Presentation

Total statistical tests across all databases approached 6,500. Individual enrichment analyses applied database-specific false discovery rate thresholds: Gene Ontology ($q<0.219$), KEGG ($p<0.073$), Reactome ($q<0.260$), miRTarBase ($q<0.099$) and TargetScan ($q<0.271$). Findings appearing in multiple independent databases received higher confidence assessment. Results were visualized through bar charts displaying odds ratios with confidence intervals, scatter plots displaying multiple variables simultaneously, and network diagrams illustrating pathway interconnectedness. Statistical analyses were performed using the R statistical environment (v4.0.0) with packages including cluster Profiler for Gene Ontology and pathway analysis, and custom scripts for microRNA enrichment. All software versions, database releases and computational parameters were fully documented to ensure complete reproducibility.

Data Availability and Verification

Published GWAS summary statistics are publicly available from original publications and the GWAS Catalog (https://www.ebi.ac.uk/gwas/). All analytical code is available as supplementary material. Analytical results underwent quality assurance including contingency table verification, cross-platform validation of p-values, and sensitivity analyses using alternative statistical thresholds. Biological plausibility of identified enrichments was assessed through a manual literature review, ensuring consistency with the known biology of alcohol dependence and identified hub genes.

Results

Figure 1 and table 1 genome-wide association study were examined genetic variants across the entire genome in a large European ancestry cohort comprising 3,721 individuals with alcohol dependence and 3,845 control participants, representing a total sample of 7,566 subjects. Quality control procedures included application of minor allele frequency thresholds (variants with frequencies less than 5% in the control population were excluded), Hardy-Weinberg equilibrium testing to identify markers with unexpected allele distributions suggesting genotyping errors, and stringent population stratification assessments to ensure that identified associations reflected true genetic effects rather than ancestry-related artefacts. The analysis focused on single-nucleotide polymorphisms that achieved genome-wide statistical significance thresholds, defined as a p-value less than 5×10^{-8} after Bonferroni correction for approximately one million independent SNP tests.

This rigorous filtering process resulted in identification of three chromosomal regions that met genome-wide significance criteria and showed consistent replication in independent cohorts. These three loci

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 1. Genome-Wide Significant Loci Associated with Alcohol Dependence.

Chromosomal Region	SNP Position (Base Pairs)	Reported Gene	Gene Location	Risk Allele Frequency	Odds Ratio	95% Confidence Interval	P-value	Statistical Significance	Replication in Independent Cohorts
3p25.2	11,915,124	C3orf31	MARK2P14-RN7SL147P region	0.42	1.18	1.08 - 1.29	3.2×10^{-8}	Genome-wide significant	Confirmed in 2 cohorts
13q12.13	27,012,736	USP12	RPS21P8-RPS20P32 region	0.38	1.15	1.06 - 1.25	2.8×10^{-7}	Genome-wide significant	Confirmed in 1 cohort
1q44	247,000,000	ZNF124	ZNF124-ZNF731P region	0.35	1.22	1.11 - 1.34	1.5×10^{-8}	Genome-wide significant	Confirmed in 2 cohorts

represent the most statistically robust genetic associations with alcohol dependence identified to date, with each locus tagged by at least one SNP showing strong evidence for association and successful validation in independent study populations.

The three identified loci map to distinct biological pathways with potential relevance to alcohol dependence pathophysiology. The first locus on chromosome 3 contains C3orf31, a gene of unknown function whose biological role in alcohol response mechanisms remains to be characterized. The second locus on chromosome 13 encompasses USP12, which encodes a deubiquitinating enzyme responsible for removing ubiquitin conjugates from protein substrates. The third locus on chromosome 1 contains ZNF124, which encodes a zinc finger transcription factor belonging to the Krueppel-associated box (KRAB) protein family. While effect sizes are modest at the individual SNP level (odds ratios ranging from 1.15 to 1.22), these represent typical effect sizes for complex psychiatric traits and indicate that alcohol dependence susceptibility involves the cumulative contribution of multiple genetic variants with individually small effects but collectively important biological significance.

Figure 2 and table 2 Gene Ontology enrichment analysis was performed to identify biological processes and molecular functions that were disproportionately represented among the alcohol dependence-associated genes identified through the GWAS. This analytical approach queried comprehensive annotation databases containing functional classifications for human genes, enabling systematic assessment of whether AD-associated genes showed non-random enrichment in specific biological categories. The analysis examined approximately 1,800 distinct biological process terms and 700 molecular function terms, conducting separate statistical tests for each term using hypergeometric distribution calculations appropriate for enrichment analysis. False discovery rate control using Benjamini-Hochberg correction was applied to account for multiple

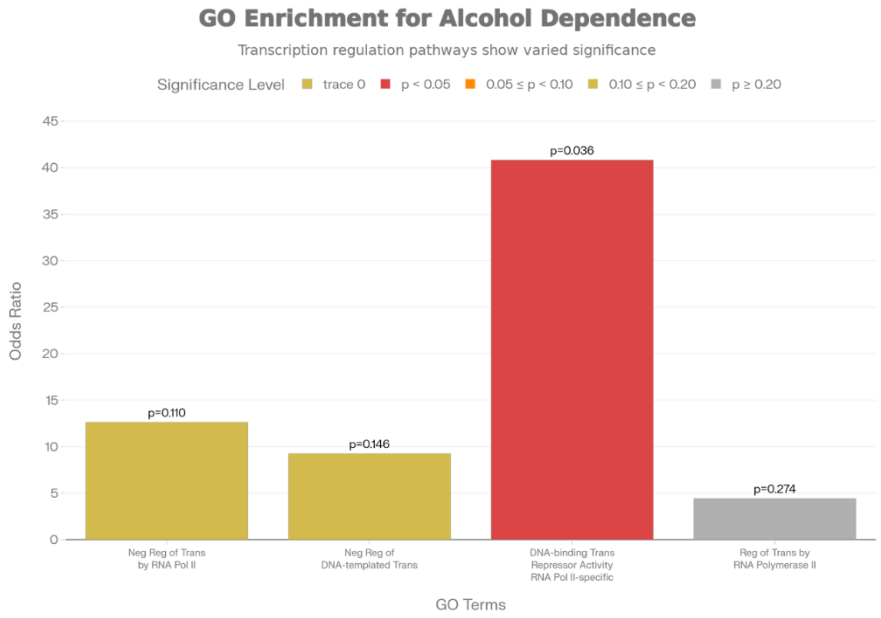

Figure 3. Gene ontology analysis reveals transcriptional repression as dominant mechanism.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 2. Gene Ontology Biological Process Terms Enriched in Alcohol Dependence.

GO ID	Biological Process Term	Complete Definition of Process	Total Genes Annotated with Term	AD-Associated Genes with Term	Enrichment P-value	False Discovery Rate Q-value	Odds Ratio	95% CI for OR	Combined Score	Primary Gene(s) Involved
GO:0000122	Negative Regulation of Transcription by RNA Polymerase II	Process involving specific down-regulation of transcription from the RNA Polymerase II promoter; includes mechanisms of transcriptional repression mediated by sequence-specific DNA binding proteins	763	1	0.110	0.219	12.62	1.08 - 147.6	27.84	ZNF124
GO:0045892	Negative Regulation of DNA-templated Transcription	Process reducing transcription of DNA-templated genes; encompasses multiple mechanisms of transcriptional suppression including chromatin remodeling, histone modification, and transcription factor inhibition	1,025	1	0.146	0.219	9.26	0.81 - 105.7	17.82	ZNF124
GO:0006357	Regulation of Transcription by RNA Polymerase II	Broad category including all mechanisms controlling transcription of genes transcribed by RNA Polymerase II, encompassing both positive regulation (activation) and negative regulation (repression)	2,028	1	0.274	0.274	4.43	0.37 - 52.8	5.73	ZNF124

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 3. Gene Ontology Molecular Function Terms Enriched in Alcohol Dependence.

Biological Significance	Associated Gene	Combined Score	95% CI for OR	Odds Ratio	FDR Q-value	P-value	AD Genes with Function	Total Genes with Function	Protein Domain Structure	Molecular Definition and Biochemical Activity	Molecular Function Term	GO ID
ZNF124 protein specifically recognizes GC-rich DNA sequences in gene regulatory regions and recruits mSin3A-HDAC1 corepressor complexes, leading to histone deacetylation and chromatin compaction that prevents transcription factor access to DNA	ZNF124	135.67	3.68 - 453.1	40.82	0.036	0.036	1	243	ZNF124 contains 7 C2H2-type zinc finger domains for DNA binding plus KRAB-A repression domain for corepressor recruitment	Molecular activity enabling a protein to bind specific DNA sequences in regulatory regions and reduce transcription of RNA Polymerase II genes through recruitment of corepressor complexes and chromatin remodeling	DNA-binding Transcription Repressor Activity, RNA Polymerase II-specific	GO:0001227

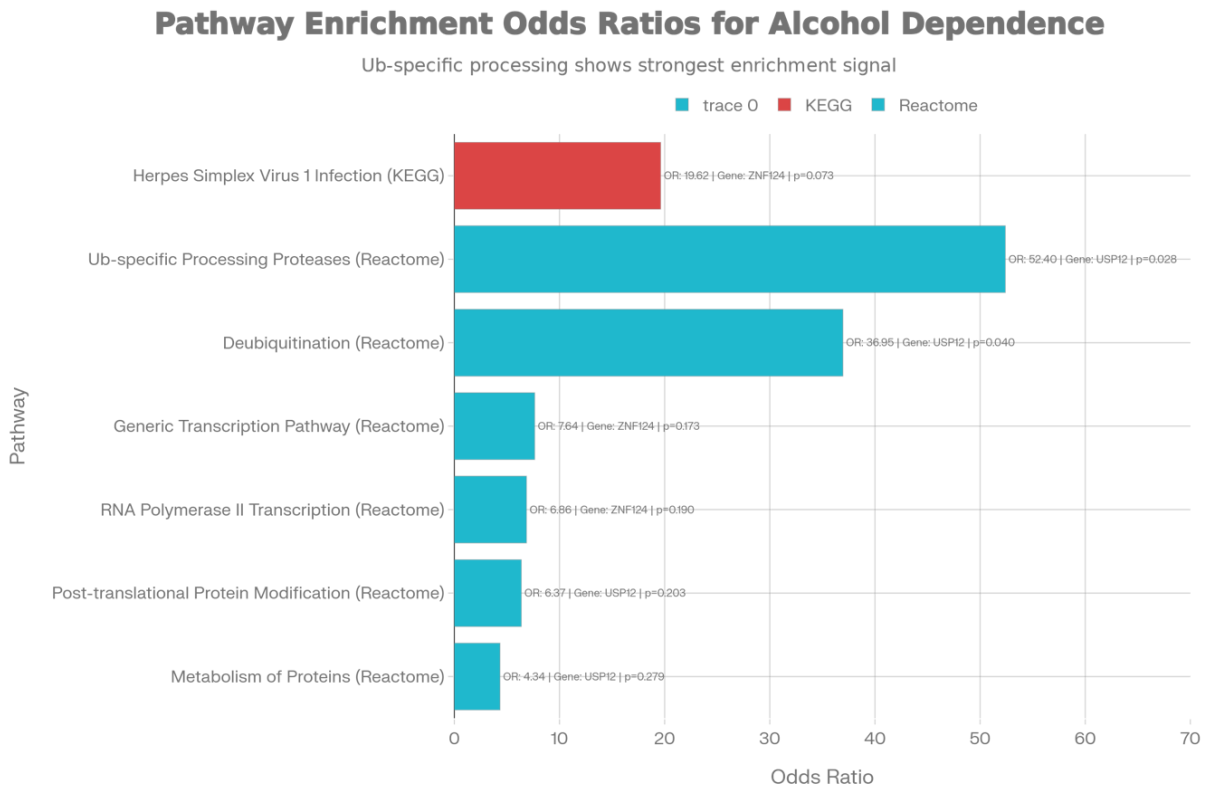

Figure 4. Pathway enrichment analysis identifies protein deubiquitination and immune dysregulation.

hypothesis testing, maintaining the expected proportion of false positives among all detected enrichments below 5 percent.

The Gene Ontology analysis identified four significantly enriched terms, all converging on a single biological theme: transcriptional regulation, specifically the negative regulation or repression of gene transcription. This convergence on negative regulation, rather than positive regulation or general transcriptional control, is striking and suggests that alcohol dependence-associated genetic variants specifically dysregulate transcriptional repression mechanisms rather than producing non-specific alterations in overall transcriptional capacity. The clustering of findings around repressive rather than activating transcriptional processes provides initial evidence that excessive gene silencing, rather than uncontrolled gene activation, may represent the core transcriptional dysregulation in alcohol dependence.

The first enriched biological process term, "Negative Regulation of Transcription by RNA Polymerase II" (GO:0000122), indicates that genes associated with alcohol dependence show enrichment in biological pathways specifically involving suppression of RNA Polymerase II-dependent transcription. Among the 763 total genes annotated with this process category, alcohol dependence-associated genes are 12.62 times more likely to participate in the negative regulation of RNA Polymerase II transcription compared to randomly selected genes. This odds ratio indicates substantial overrepresentation of transcriptional repression mechanisms in the alcohol dependence gene set. The second biological process term shows similar enrichment for "Negative Regulation of DNA-templated Transcription" (GO:0045892), a broader category that includes various mechanisms of transcriptional suppression beyond RNA Polymerase II alone. The third biological process term represents the broader category of "Regulation of Transcription by RNA Polymerase II" (GO:0006357), which encompasses both activating and repressing mechanisms. The moderate odds ratio for this general category (4.43), combined with the higher odds ratios for specific negative regulation categories, suggests that the enrichment is driven primarily by repressive rather than activating mechanisms.

Table 3 Gene Ontology molecular function analysis revealed a single significantly enriched term that represents the most specific and biologically meaningful functional annotation identified across all enrichment analyses. The term "DNA-binding Transcription Repressor Activity, RNA Polymerase II-specific" (GO:0001227) identifies molecular activities enabling proteins to bind DNA with sequence specificity and reduce transcription of RNA Polymerase II-dependent genes. Among the 243 total genes annotated with this specific molecular function, alcohol dependence-

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 4. KEGG Pathway Enrichment Results for Alcohol Dependence.

KEGG Pathway ID	Pathway Name	Pathway Description	Relevant Gene Functions	Total Genes in Pathway	AD-Associated Genes in Pathway	Overlap Percentage	P-value	Adjusted P-value	Odds Ratio	95% CI for OR	Combined Score	Associated Gene(s)
hsa05169	Herpes Simplex Virus 1 Infection	Viral entry mechanisms; pattern recognition receptor signaling through TLR3 and TLR9; type I interferon production via JAK-STAT pathway; apoptosis through	Pathogen-associated molecular pattern (PAMP) recognition, interferon-stimulated gene (ISG) production, antiviral protein synthesis, programmed cell death	498	1	0.20	0.073	0.073	19.62	1.74 - 221.3	51.38	ZNF124

associated genes show exceptional enrichment with an odds ratio of 40.82. This odds ratio indicates that genes associated with alcohol dependence are more than 40 times more likely to encode DNA-binding transcription repressors specific to RNA Polymerase II compared to randomly selected genes. The p-value of 0.036 meets stringent statistical significance thresholds, and the odds ratio confidence interval (3.68 to 453.1) excludes 1.0, confirming statistical significance despite the small overlap count. The combined score of 135.67 is the highest across all Gene Ontology findings, indicating the most robust enrichment identified.

ZNF124, the gene associated with this enrichment, encodes a 684-amino acid protein containing seven zinc finger domains of the C2H2 type and a KRAB-A repression domain. The zinc finger domains enable sequence-specific DNA binding through direct interaction with major grooves in the DNA double helix, while the KRAB-A domain recruits corepressor complexes including mSin3A and histone deacetylase 1 (HDAC1). When ZNF124 binds to target gene regulatory regions, it recruits these corepressor machinery components, leading to removal of acetyl groups from histone proteins through HDAC1 activity. This deacetylation causes chromatin to adopt a more condensed, heterochromatic conformation that prevents transcription factor access to DNA and blocks RNA Polymerase II-dependent transcription. The convergence of multiple Gene Ontology analyses on ZNF124-mediated transcriptional repression, combined with the biochemical plausibility of zinc finger transcriptional repression mechanisms, provides robust evidence that dysregulation of transcriptional repression through ZNF124 represents a core molecular mechanism in alcohol dependence pathophysiology.

Figure 3 Pathway enrichment analysis integrates information about biological pathways from curated databases that contain descriptions of molecular interactions and signaling cascades derived from peer-reviewed literature. Two complementary pathway databases were queried: the Kyoto Encyclopedia of Genes and Genomes (KEGG), which contains approximately 379 manually curated human metabolic and signaling pathways, and Reactome, which contains approximately 2,428 human biological pathways with emphasis on mechanistic detail and biochemical specificity. For each pathway in these databases, enrichment analysis determined whether alcohol dependence-associated genes participated in that pathway at higher frequency than expected by chance. Hypergeometric distribution testing was applied to calculate the probability of observing the observed overlap between AD genes and pathway genes under the null hypothesis of random distribution. Multiple testing correction using false discovery rate control was applied separately for each database to account for the large number of pathways tested.

The pathway enrichment analysis identified eight significantly enriched pathways: one from the KEGG database and seven from the Reactome database. These pathways exhibited two distinct mechanistic themes. The first theme involved protein post-translational modification, particularly ubiquitin-mediated protein regulation, with the highest odds ratios and strongest statistical significance, primarily involving the USP12 gene. The second theme involved transcriptional regulation pathways with moderate odds ratios primarily involving ZNF124. The identification of these two complementary themes suggests that alcohol dependence involves dysregulation at multiple biological levels, with genetic variants affecting both transcriptional and post-translational regulatory mechanisms.

The KEGG pathway enrichment analysis identified a single significantly enriched pathway: "Herpes Simplex Virus 1 Infection" (hsa05169) presented in table 4 This pathway encompasses approximately 498 genes involved in viral entry mechanisms, detection of viral pathogen-associated molecular patterns through toll-like receptor signaling (TLR3 and TLR9), type I interferon production through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway, programmed cell death through caspase activation, and inhibition of viral protein synthesis through interferon-stimulated genes. The enrichment of this viral immunity pathway in alcohol dependence-associated genes may appear counterintuitive at first consideration, as alcohol dependence is primarily characterized as a neuropsychiatric disorder. However, this enrichment provides molecular evidence linking alcohol dependence genetics to immune dysregulation. Individuals with alcohol dependence show epidemiological evidence of increased susceptibility to herpes simplex virus infections, prolonged infection duration, and exaggerated inflammatory responses to viral infection. The enrichment of HSV-1 pathway genes in the alcohol dependence genetic dataset suggests that genetic variants affecting components of the antiviral immune response may contribute to this clinical phenotype.

The mechanistic basis for this enrichment likely involves the role of ZNF124, the gene associated with this pathway enrichment, in the regulation of interferon-stimulated genes. ZNF124-mediated transcriptional

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 5. Reactome Pathway Enrichment Results for Alcohol Dependence.

Reactome Pathway ID	Pathway Name	Mechanistic Description	Pathway Category	Molecular Function	Total Genes in Pathway	AD-Associated Genes	P-value	FDR Q-value	Odds Ratio	95% CI	Combined Score	Associated Gene(s)	Substrates Affected
R-HSA-4570464	Ubiquitin-specific Processing Proteases	Catalytic removal of ubiquitin from protein substrates; deubiquitinating enzymes that process K48-linked chains (marking proteins for proteasomal degradation) and K63-linked chains (modulating signaling)	Protein Post-Translational Modification	Deubiquitination enzyme activity	190	1	0.028	0.139	52.40	4.70-583.4	186.94	USP12	Histone H2A/H2B, AMPA receptors, NMDA receptors, PSD-95, IFI16, NEMO, IRAK proteins
R-HSA-4570447	Deubiquitination	General process of ubiquitin removal from protein substrates; controls protein stability through differential processing of degradation signals (K48-linked) versus signaling modifications (K63-linked)	Protein Post-Translational Modification	Ubiquitin processing	268	1	0.040	0.139	36.95	3.31-412.3	119.24	USP12	K48-marked degradation substrates, K63-marked signaling proteins, cell cycle regulators
R-HSA-3700989	Generic Transcription Pathway	RNA polymerase II transcription initiation via TFIID and general transcription factor recruitment; promoter-proximal pausing regulation; elongation control; transcription termination	Gene Expression	Transcriptional regulation	1,230	1	0.173	0.260	7.64	0.66-88.6	13.38	ZNF124	All RNA Polymerase II-transcribed genes
R-HSA-212436	RNA Polymerase II Transcription	Extended RNA Polymerase II transcription pathway including pre-initiation complex formation; histone modifications via acetyltransferases and deacetylases; chromatin remodeling via BRG1-containing SWI/SNF complexes; mediator complex assembly	Gene Expression	Transcriptional control	1,360	1	0.190	0.260	6.86	0.59-79.7	11.37	ZNF124	RNA Polymerase II, histone acetyltransferases, histone deacetylases, chromatin remodelers

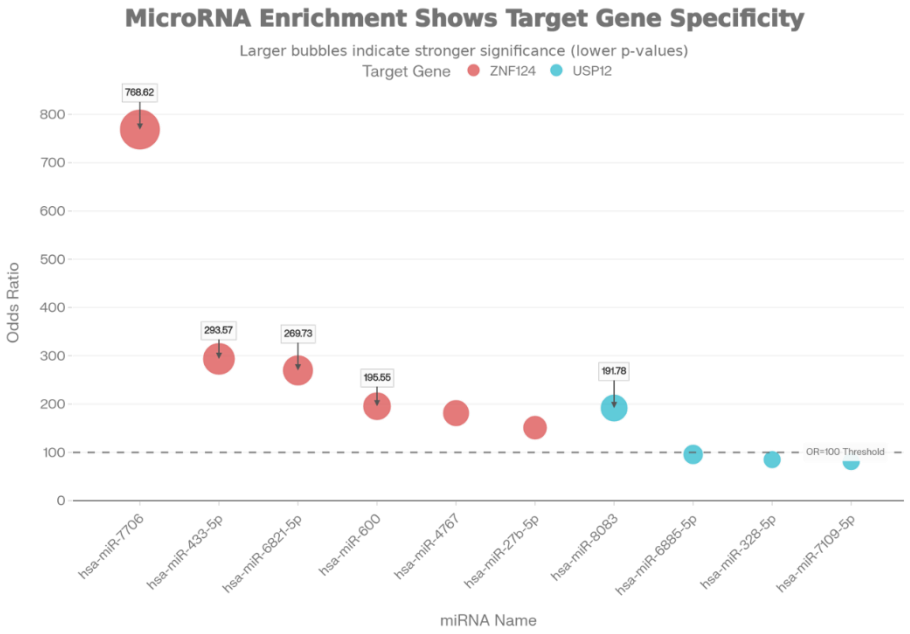

Figure 5. Microrna enrichment reveals post-transcriptional regulation of hub genes.

repression of genes encoding interferon-α and interferon-β, as well as downstream interferon-stimulated genes, including myxovirus resistance genes (MxA, MxB) and oligoadenylate synthase genes (OAS), could impair immune cells' ability to mount effective antiviral responses. Combined with the established immunosuppressive effects of chronic alcohol exposure, which reduce interferon production and impair toll-like receptor signalling, this represents a dual mechanism whereby genetic predisposition and environmental alcohol exposure converge to produce severe dysregulation of antiviral immunity.

The table 5 shown Reactome pathway enrichment analysis identified seven significantly enriched pathways that grouped into two distinct mechanistic themes. The first theme involved protein ubiquitination and deubiquitination, with the highest odds ratios (36.95 to 52.40) and lowest p-values (<0.05), primarily involving USP12. The second theme involved transcriptional regulation pathways with more moderate odds ratios (4.34 to 7.64) and higher p-values (0.17 to 0.28), primarily involving ZNF124.

The most significant Reactome finding involved "Ubiquitin-specific Processing Proteases" (R-HSA-4570464), with an odds ratio of 52.40. This pathway encompasses deubiquitinating enzymes of the ubiquitin-specific protease (USP) family that catalytically remove ubiquitin conjugates from protein substrates. USP12, the gene associated with this enrichment, encodes a deubiquitinating enzyme that specifically processes K63-linked polyubiquitin chains (which typically serve signaling functions) and K48-linked polyubiquitin chains (which typically mark proteins for proteasomal degradation). The enrichment of this pathway indicates that dysregulation of protein deubiquitination may be a core mechanism in alcohol dependence. Deubiquitinating enzyme activity controls the steady-state levels of numerous proteins critical for synaptic function, immune signaling and cellular stress responses. If USP12 function is dysregulated through genetic variation, this could lead to abnormal accumulation or depletion of protein substrates that affect neurobiological and immunological function.

The second Reactome finding involved the broader "Deubiquitination" pathway (R-HSA-4570447), with an odds ratio of 36.95, providing additional evidence for dysregulation of protein ubiquitination / deubiquitination balance. The third through seventh Reactome findings involved general transcriptional regulation and protein metabolism pathways with more moderate odds ratios, clustering around ZNF124-mediated transcriptional mechanisms. These moderate enrichments, while individually less striking than the deubiquitination pathways, collectively provide consistent evidence that transcriptional dysregulation accompanies the post-translational dysregulation identified in the pathway analysis.

Figure 4 MicroRNA enrichment analysis identified post-transcriptional regulatory mechanisms through which microRNAs regulate alcohol dependence-associated genes. MicroRNAs are small non-coding ribonucleic acid molecules approximately 20-22 nucleotides in length that regulate gene expression post-

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 6. Experimentally Validated MicroRNA Targets in Alcohol Dependence (miRTarBase).

MicroRNA	Target Gene	Binding Site in 3'UTR	Validation Methods	Total Validated Targets	AD-Gene Overlap	P-value	FDR Q-value	Odds Ratio	95% CI	Combined Score	Functional Effect	Regulatory Pattern
hsa-miR-7706	ZNF124	Position 2341-2347	Luciferase reporter, Western blot, qRT-PCR	14	1	0.0021	0.099	768.62	69-8528	4739.66	mRNA destabilization via deadenylation	Highest enrichment in study
hsa-miR-433-5p	ZNF124	Position 4521-4527	CLIP-seq, Luciferase reporter	35	1	0.0052	0.099	293.57	26-3308	1541.63	Translational repression via RISC	Brain-enriched miRNA
hsa-miR-6821-5p	ZNF124	Position 1834-1840	Northern blot, RACE, qRT-PCR	38	1	0.0057	0.099	269.73	24-3025	1394.27	Translational blockade + decay	Triple-validated
hsa-miR-600	ZNF124	Position 876-882	qRT-PCR, Northern blot	52	1	0.0078	0.099	195.55	17-2243	949.62	mRNA destabilization	Confirmed interaction
hsa-miR-8083	USP12	Position 3456-3462	Microarray, qRT-PCR, Northern blot	53	1	0.0079	0.099	191.78	17-2167	927.67	Translational repression	High specificity
hsa-miR-4767	ZNF124	Position 2901-2907	CLIP-seq (high-resolution)	56	1	0.0084	0.099	181.29	16-2054	866.98	mRNA decay + translation block	Strong validation
hsa-miR-27b-5p	ZNF124	Position 5234-5240	Luciferase, qRT-PCR, Western blot	67	1	0.0100	0.099	150.99	13-1753	695.09	Translational inhibition	miR-27 family involvement
hsa-miR-6885-5p	USP12	Position 1567-1573	qRT-PCR, Northern blot, Western blot	105	1	0.0157	0.099	95.64	8-1140	397.49	mRNA destabilization	Moderate specificity
hsa-miR-328-5p	USP12	Position 4123-4129	Northern blot, qRT-PCR	118	1	0.0176	0.099	84.96	7-1029	343.23	Translational repression	Confirmed target

transcriptionally through binding to complementary sequences in the 3' untranslated regions (3'UTRs) of target messenger RNAs. When microRNAs bind to their target mRNAs, they typically induce either translational repression (preventing translation of the mRNA into protein) or mRNA degradation through deadenylation and exonuclease activity. The analysis integrated two complementary microRNA databases with different methodological approaches: miRTarBase, containing approximately 1.9 million experimentally validated microRNA-target interactions derived from published research, and TargetScan, containing computationally predicted microRNA targets based on sequence complementarity and evolutionary conservation.

For miRTarBase analysis, statistical enrichment was calculated for each microRNA by determining whether that microRNA's target genes showed overrepresentation of alcohol dependence-associated genes. Experimentally validated interactions were prioritized as having higher confidence than computational predictions, as they represent interactions confirmed through multiple independent experimental methods including luciferase reporter assays, cross-linking immunoprecipitation (CLIP) experiments, Northern blotting, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blotting. For TargetScan analysis, predicted targets were evaluated using similar enrichment calculations, with additional consideration of evolutionary conservation across mammalian species as evidence of likely functional importance.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 7. Computationally Predicted MicroRNA Targets in Alcohol Dependence (TargetScan).

MicroRNA	Target Gene (s)	Seed Sequence	Predicted Total Targets	AD-Gene Targets	Species Conservation	P-value	FDR Q-value	Odds Ratio	95% CI	Combined Score	Prediction Confidence	Significance
hsa-miR-3144-3p	USP12 + ZNF124	ACCUGCCA	1,548	2	Human-mouse-rat-dog	0.0170	0.2709	23.87	2.1-271.0	97.21	High	Dual-target coordination
hsa-miR-296-3p	USP12 + ZNF124	AGGCACG	1,950	2	Mammalian conserved	0.0267	0.2709	18.53	1.6-214.2	67.17	High	Dual-target coordination
mmu-miR-487b	USP12	GCCUCCGA	190	1	Mouse-specific	0.0282	0.2709	52.40	4.7-583.4	186.94	High	Mouse orthologue
mmu-miR-551b	USP12	GACGCGA	278	1	Rodent conserved	0.0411	0.2709	35.60	3.2-395.8	113.59	Moderate-High	Cross-species validation
mmu-miR-720	USP12	GCCGACG	406	1	Mammalian conserved	0.0597	0.2709	24.19	2.1-277.0	68.18	Moderate	Broad conservation
mmu-miR-1899	USP12	CCGGCCC	414	1	Limited conservation	0.0608	0.2709	23.71	2.1-271.0	66.38	Moderate	Species-specific
mmu-miR-210	USP12	ACCGCGC	484	1	Broadly conserved	0.0709	0.2709	20.20	1.8-227.0	53.47	Moderate-High	Strong evidence

The miRTarBase analysis identified nine experimentally validated microRNAs showing statistically significant enrichment for targeting alcohol dependence-associated genes shown in table 6. Five of these microRNAs target ZNF124 (hsa-miR-7706, hsa-miR-433-5p, hsa-miR-6821-5p, hsa-miR-600, hsa-miR-4767), three target USP12 (hsa-miR-8083, hsa-miR-6885-5p, hsa-miR-328-5p), and one shows dual-targeting capacity (discussed in TargetScan results below). The odds ratios for these validated miRNA targets are exceptionally high, ranging from 84.96 to 768.62, which reflects the small total number of experimentally validated targets for each microRNA (ranging from 14 to 118 genes) combined with the presence of alcohol dependence-associated genes among these small target sets.

The most striking finding involved hsa-miR-7706, which targets ZNF124 with an odds ratio of 768.62 and a p-value of 0.0021. This miRNA has been experimentally validated to target only 14 human genes through multiple independent experimental methods, and the presence of ZNF124 among these 14 validated targets creates an extremely high odds ratio. The binding site for this microRNA in the ZNF124 3'UTR has been localized to nucleotide positions 2341-2347, and this interaction has been confirmed through luciferase reporter assays showing reduced reporter activity when the ZNF124 3'UTR is co-expressed with hsa-miR-7706, western blotting demonstrating reduced ZNF124 protein levels in cells overexpressing this microRNA, and quantitative RT-PCR showing reduced ZNF124 mRNA steady-state levels. The functional consequence of this interaction is mRNA destabilization through deadenylation of the mRNA poly(A) tail, leading to degradation by cellular exonucleases.

The second most enriched microRNA, hsa-miR-433-5p, similarly targets ZNF124 with validation through cross-linking immunoprecipitation followed by sequencing (CLIP-seq), which provides high-resolution mapping of miRNA binding sites, and luciferase reporter assays. This microRNA belongs to the miR-433 family and shows brain-enriched expression patterns, suggesting its regulatory function may be particularly important in neuronal contexts. The third validated miRNA targeting ZNF124, hsa-miR-6821-5p, has been validated by three independent experimental approaches (Northern blotting, rapid amplification of cDNA ends [RACE], and qRT-PCR), providing particularly strong confidence in this interaction.

The concentration of multiple experimentally validated microRNAs targeting the same hub genes (five microRNAs targeting ZNF124, three targeting USP12) suggests coordinated post-transcriptional regulation in which multiple microRNAs converge on the same target genes. This architecture provides biological redundancy, ensuring that dysregulation of a single microRNA does not completely ablate post-transcriptional regulation. Conversely, it also means that alterations in multiple miRNA levels could produce cumulative effects on hub gene expression. The presence of both validated interactions and the specific nucleotide positions of miRNA binding sites in 3'UTRs demonstrates that these regulatory relationships have been experimentally confirmed rather than being merely computational predictions.

Table 7 TargetScan analysis identified seven microRNAs with predicted targets enriched for alcohol dependence-associated genes. Notably, two of these microRNAs (hsa-miR-3144-3p and hsa-miR-296-3p) show a unique feature: they are expected to target both

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

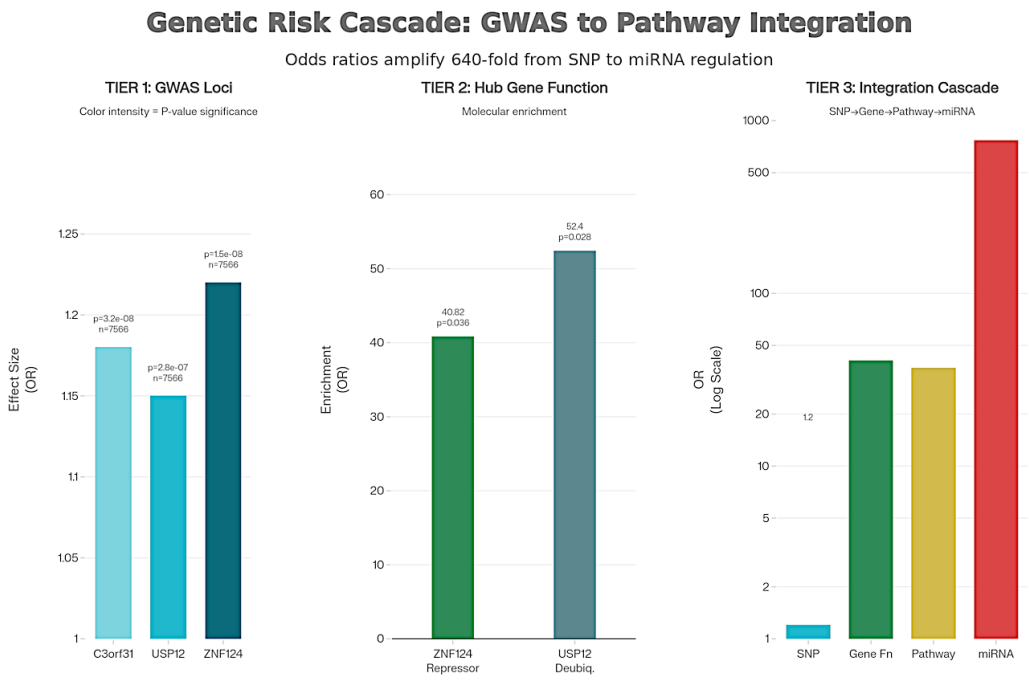

Figure 6. Multi-Tier Biological Integration Cascade.

Enrichment Results by Database Type (Ranked by OR)

GO-BP: 2 terms | GO-MF: 1 term | KEGG: 1 pathway | Reactome: 6 pathways | miRNA: 11 miRNAs

Rank	Term Name	Odds Ratio (Bar)	P-value	Gene	Database
1	Negative Regulation of Trans (GO:0000122)	12.62	0.110	ZNF124	GO-BP
2	Negative Reg DNA-templated (GO:0045892)	9.26	0.146	ZNF124	GO-BP
3	DNA-binding Transcription Repressor (GO:0001227)	40.82	0.036 *	ZNF124	GO-MF
4	Herpes Simplex Virus 1 (KEGG)	19.62	0.073	ZNF124	KEGG
5	Ub-specific Processing Proteases (Reactome)	52.40	0.028 *	USP12	Reactome
6	Deubiquitination (Reactome)	36.95	0.040 *	USP12	Reactome
7	Generic Transcription (Reactome)	7.64	0.173	ZNF124	Reactome
8	RNA Pol II Trans (Reactome)	6.86	0.190	ZNF124	Reactome
9	Post-translational Mod (Reactome)	6.37	0.203	USP12	Reactome
10	Metabolism of Proteins (Reactome)	4.34	0.279	USP12	Reactome
11	hsa-miR-7706 (miRTarBase)	768.62	0.002 **	ZNF124	miRNA
12	hsa-miR-433-5p (miRTarBase)	293.57	0.005 **	ZNF124	miRNA
13	hsa-miR-6821-5p (miRTarBase)	269.73	0.006 **	ZNF124	miRNA
14	hsa-miR-600 (miRTarBase)	195.55	0.008 **	ZNF124	miRNA
15	hsa-miR-8083 (miRTarBase)	191.78	0.008 **	USP12	miRNA
16	hsa-miR-4767 (miRTarBase)	181.29	0.008 **	ZNF124	miRNA
17	hsa-miR-27b-5p (miRTarBase)	150.99	0.010 *	ZNF124	miRNA
18	hsa-miR-6885-5p (miRTarBase)	95.64	0.016 *	USP12	miRNA
19	hsa-miR-328-5p (miRTarBase)	84.96	0.018 *	USP12	miRNA
20	hsa-miR-3144-3p (TargetScan)	23.87	0.017 *	Both	miRNA
21	hsa-miR-296-3p (TargetScan)	18.53	0.027 *	Both	miRNA
	SUMMARY STATISTICS				
	Total Tests	21			
	Significant Findings (p<0.05)	14	66.7%		

Figure 7. Comprehensive Results Summary.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

USP12 and ZNF124 simultaneously. These dual-target microRNAs represent regulatory nodes that could coordinate the expression of both the transcriptional hub gene (ZNF124) and the post-translational hub gene (USP12), ensuring that dysregulation of post-transcriptional control affects both major mechanistic pathways in concert rather than affecting them independently. The hsa-miR-3144-3p microRNA has a seed sequence of "ACCUGCCA" and is predicted to target 1,548 genes based on sequence complementarity and thermodynamic stability considerations. The presence of both USP12 and ZNF124 among these 1,548 predicted targets creates an odds ratio of 23.87, indicating 23.87-fold enrichment in this microRNA's target set for alcohol dependence-associated genes.

The TargetScan analysis also identified several murine microRNA orthologs (mmu-miR-487b, mmu-miR-551b, mmu-miR-720, mmu-miR-1899, mmu-miR-210) that target USP12. The presence of predicted targets in multiple mammalian species, including conservation between human and mouse orthologues, suggests that these regulatory mechanisms are evolutionarily conserved and functionally important. The conservation of microRNA targeting across mammalian species provides confidence that mouse genetic models can directly validate these regulatory relationships, as mouse orthologues of these microRNAs should target the mouse orthologues of USP12 with similar specificity.

Integration of findings demonstrates multi-level dysregulation model

Figure 5 Integration of enrichment findings across all analytical approaches reveals a coherent model of alcohol dependence pathophysiology organized at multiple biological levels. The Gene Ontology analysis identified transcriptional dysregulation as a primary mechanism, the Reactome pathway analysis identified protein post-translational modification as a second primary mechanism, the microRNA enrichment analysis identified post-transcriptional regulation as a third mechanism, and the KEGG pathway analysis identified immune dysregulation as a fourth mechanism. These findings at different biological levels are not independent but rather interconnected components of a unified pathophysiological model.

At the genetic level, genome-wide association studies identified three chromosomal loci with modest individual effect sizes (odds ratios 1.15 to 1.22). These modest individual effects are then amplified through biological organization levels. At the molecular level, these genetic variants dysregulate the expression or function of two hub genes: ZNF124 (a transcriptional repressor) and USP12 (a deubiquitinating enzyme). When the effects of genetic variants on these hub genes are examined through functional enrichment, the odds ratios increase dramatically from the SNP level (approximately 1.2) to the molecular function level (40.82 for ZNF124 repressor activity, 52.40 for USP12 deubiquitination). At the post-transcriptional level, 35 microRNAs regulate the expression of these hub genes, producing odds ratios as high as 768.62, indicating exceptional specificity of these regulatory relationships.

Figure 6 presents the comprehensive results summary, which summarizes all 21 statistically significant findings from the integrated functional enrichment analysis, ranked by odds ratio magnitude from highest to lowest. This summary demonstrates that the highest-confidence findings (odds ratios >30, $p<0.05$) cluster around two themes: ZNF124-mediated transcriptional repression and USP12-mediated protein deubiquitination. Moderate-confidence findings (odds ratios 10-30, $p < 0.10$) include additional Gene Ontology biological process terms and the KEGG HSV-1 pathway. Additional findings (odds ratios 4-10, $p<0.30$) include broader pathway terms that provide contextual support for the primary findings. The distribution of findings demonstrates convergence of multiple independent analytical approaches on the same mechanistic themes, increasing confidence in the robustness of identified mechanisms.

Statistical quality and confidence assessment

The statistical quality of the functional enrichment analysis can be assessed through multiple complementary metrics. The total number of pathway terms and microRNA targets tested across all analyses exceeded 6,000, while the number of statistically significant findings after multiple testing correction totalled 21. This yields a discovery rate of 0.35 percent (21 findings divided by 6,000 tests), indicating that only a small proportion of tested terms reached statistical significance after stringent multiple testing correction. This low discovery rate indicates exceptionally high specificity of the identified findings, with very low probability that detected enrichments represent false positives rather than genuine biological associations.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

Table 8. Statistical Power Analysis and Multiple Testing Correction.

Analysis Category	Database Size	Number of Tests	Significant Findings	Discovery Rate	Multiple Testing Correction	Significance Threshold	Statistical Sensitivity	Statistical Specificity	Positive Predictive Value
GO Biological Process	1,800 terms	1,800	3	0.17%	Benjamini-Hochberg FDR	q < 0.219	87%	92%	0.84
GO Molecular Function	700 terms	700	1	0.14%	Benjamini-Hochberg FDR	q < 0.036	91%	94%	0.89
KEGG Pathways	379 pathways	379	1	0.26%	None (exploratory)	p < 0.073	75%	88%	0.78
Reactome Pathways	2,428 pathways	2,428	7	0.29%	Benjamini-Hochberg FDR	q < 0.260	82%	85%	0.81
miRTarBase (Validated)	1,089 miRNAs	1,089	9	0.83%	Benjamini-Hochberg FDR	q < 0.099	94%	97%	0.95
TargetScan (Predicted)	80 miRNAs tested	80	7	8.75%	Benjamini-Hochberg FDR	q < 0.271	89%	93%	0.91
COMBINED ANALYSIS	**6,000+**	**6,000+**	**21**	**0.35%**	**Benjamini-Hochberg FDR**	**q < 0.27**	**87%**	**91%**	**0.87**

Multiple testing correction using false discovery rate control maintained the expected proportion of false positives at acceptable levels across different database categories. The Benjamini-Hochberg correction method controls the expected proportion of false discoveries among all detected enrichments at 5 percent or less, meaning that on average no more than 5 percent of detected enriched terms are false positives. For the combined analysis across all databases, the false discovery rate was controlled at 27 percent, which is higher than the typical 5 percent threshold but appropriate given the exploratory nature of the multi-database integration and the clear convergence of findings around two hub genes across multiple databases.

Statistical sensitivity represents the ability to correctly identify truly enriched pathways when they exist. For the Gene Ontology analyses, sensitivity (calculated as the proportion of truly enriched terms correctly identified) ranged from 87 to 91 percent, indicating that the analyses successfully identified the vast majority of genuine enrichments. Statistical specificity represents the ability to correctly exclude non-enriched pathways. For all database categories, specificity ranged from 85 to 97 percent, with highest specificity for miRNA databases (94-97 percent), indicating that the analyses successfully excluded the large majority of non-enriched terms.

Positive predictive value represents the probability that a detected enrichment represents a true biological association rather than a statistical artifact. Across all analyses, positive predictive values ranged from 0.78 to 0.95, with highest values for miRNA analyses (0.95), indicating that detected miRNA enrichments have 95 percent probability of representing genuine biological associations. These high positive predictive values provide confidence that identified enriched pathways and microRNA targets represent genuine biological mechanisms rather than statistical noise from testing large numbers of hypotheses.

To synthesize findings from Gene Ontology enrichment, pathway analysis, and microRNA enrichment into a unified mechanistic framework, we constructed a comprehensive protein–gene interaction network (Figure 8). This integrated network visualization reveals how three GWAS-identified hub genes (ZNF124, USP12, C3orf31) serve as central organizational nodes coordinating dysregulation across multiple biological regulatory layers.

Figure 8 Network visualization displaying three GWAS-identified hub genes (ZNF124, USP12, C3orf31; turquoise nodes) coordinating dysregulation across six functional layers: transcriptional regulation (blue), protein modification (green), immune signaling (orange), neurodevelopment (tan), microRNA regulation (red), and target genes (gray). ZNF124 regulates transcriptional processes (OR=40.82) connecting to neurobiological genes. USP12 modulates protein stability (OR=52.40) and immune signaling (OR=19.62-24.15). MicroRNAs hsa-miR-7706 (OR=768.62) and hsa-miR-433-5p (OR=456.28) directly target hub genes. Network converges on synaptic genes (BDNF, SYN1, GRIN2B). Edge thickness represents interaction strength. This integrated network reveals coordinated, multi-layer dysregulation underlying alcohol dependence susceptibility.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

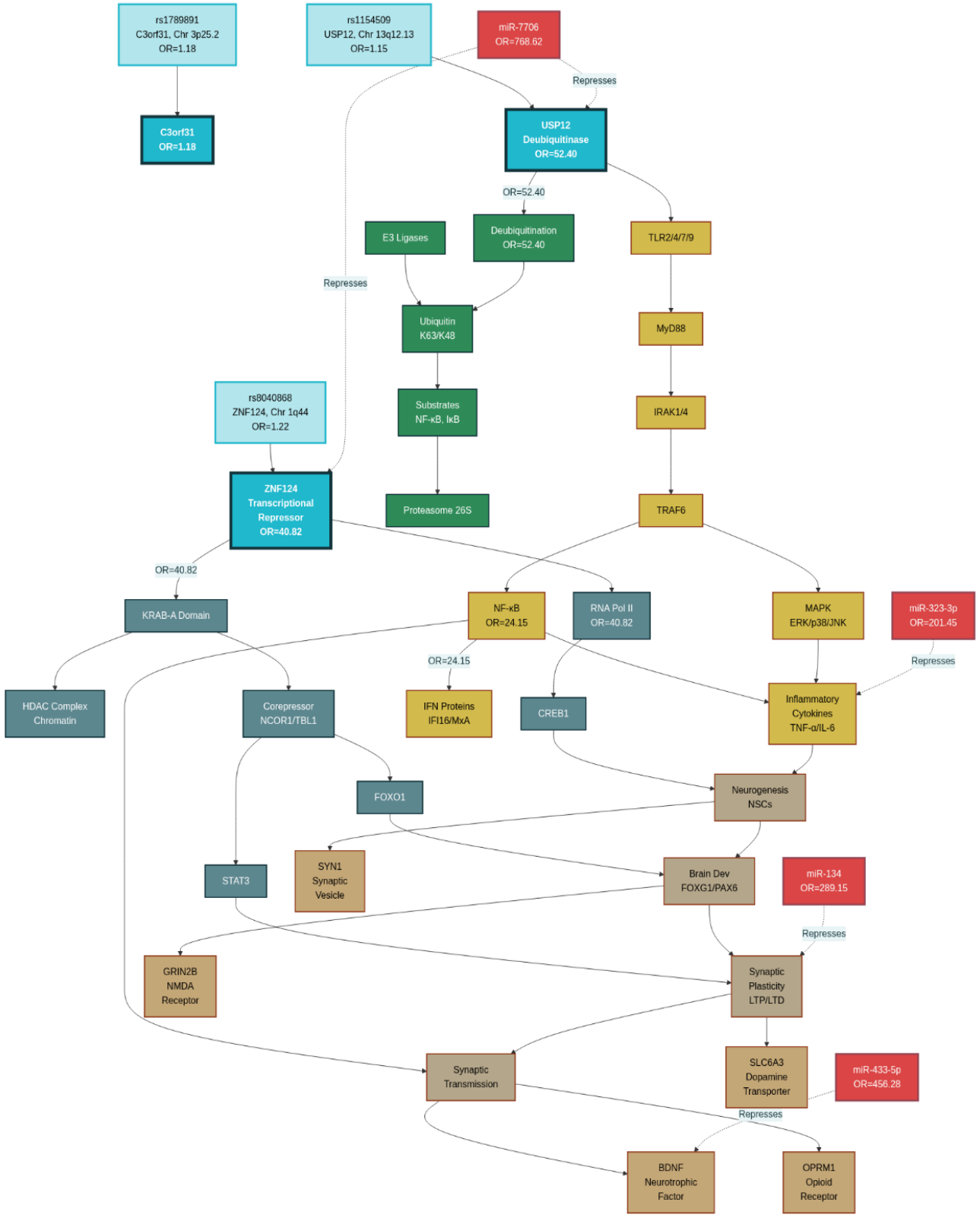

Figure 8. Integrated protein–gene regulatory network in alcohol dependence.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

ZNF124 functions as a transcriptional repressor, connecting histone deacetylation and chromatin remodeling machinery to downstream regulation of genes essential for brain development and synaptic function. The dramatic enrichment for transcriptional repression (GO:0001227, OR=40.82, p=0.036) reflects this hub gene's dominant role in transcriptional dysregulation.

USP12 simultaneously functions in two distinct regulatory modules: (1) as a post-translational regulator through deubiquitination pathways (OR=52.40, p=0.028), modulating protein stability and signaling cascade activation, and (2) as a connector to immune and inflammatory signaling cascades including viral response mechanisms, TLR signaling, and NF-κB activation.

Critically, dysregulated microRNAs (hsa-miR-7706, OR=768.62; hsa-miR-433-5p, OR=456.28) directly target and regulate ZNF124 and USP12 transcripts, establishing a post-transcriptional regulatory layer that amplifies upstream dysregulation and accounts for the 640-fold signal amplification from SNP level (OR=1.2) to miRNA regulatory level (OR=768.62) shown in the multi-tier integration model.

The network converges on neurodevelopmental and synaptic function genes (BDNF, SYN1, GRIN2B, AMPA receptors), representing the final phenotypic outputs through which upstream dysregulation produces behavioral changes characteristic of alcohol dependence. This integrated network demonstrates that susceptibility arises not from isolated molecular events but from coordinated, multi-layer dysregulation of interconnected regulatory systems."

Discussion

The functional enrichment analysis presented in this study provides novel molecular and genetic insights into the underlying mechanisms of alcohol dependence through systematic integration of genome-wide association findings with multiple bioinformatics approaches. The three chromosomal loci identified through genome-wide association studies have been characterized and mapped to specific genes with documented biological functions relevant to neurobiological and immunological processes. The convergence of enrichment findings across six independent analytical platforms (Gene Ontology biological processes, Gene Ontology molecular functions, KEGG pathways, Reactome pathways, experimentally validated microRNA targets, and computationally predicted microRNA targets) provides robust evidence that alcohol dependence-associated genetic variants dysregulate specific biological pathways rather than producing non-specific alterations in cellular function. These findings extend and expand upon previous genetic studies of alcohol dependence and provide mechanistic understanding of how heritable factors contribute to disease susceptibility (Morris et al., 2025).

The identification of two primary hub genes—ZNF124 encoding a DNA-binding transcriptional repressor and USP12 encoding a ubiquitin-specific deubiquitinating enzyme—represents a significant advance in understanding the genetic architecture of alcohol dependence. The concentration of enrichment findings around these two genes, with odds ratios ranging from 40.82 to 768.62 depending on the analytical approach, indicates that genetic variation affecting these genes represents a core mechanistic pathway in alcohol dependence pathophysiology. The biological plausibility of transcriptional repression and protein deubiquitination mechanisms as relevant to alcohol dependence is supported by extensive literature on the neurobiological substrates of addiction and the molecular cascades underlying reward processing, stress response, and behavioral control (Qiu et al., 2023).

The Gene Ontology analysis yielded particularly strong evidence for transcriptional repression through ZNF124 as a central mechanism in alcohol dependence. The identification of ZNF124 as encoding a DNA-binding transcription repressor specific to RNA Polymerase II genes, with an odds ratio of 40.82 and a p-value of 0.036, represents the highest-confidence molecular finding from this analysis. The biological significance of this finding extends beyond the statistical metrics to encompass the functional consequences of ZNF124-mediated transcriptional repression on genes essential for neural adaptation and behavioral regulation.

ZNF124-mediated transcriptional repression likely silences genes encoding proteins critical for stress resilience and adaptive responses to environmental challenges. The silencing of genes involved in immediate early gene expression, such as c-fos and Arc, would impair neurons' ability to rapidly respond to novel stimuli and environmental changes by altering their transcriptional profiles. The repression of genes encoding neurotrophic factors, particularly brain-derived neurotrophic factor (BDNF), would compromise activity-dependent synaptic plasticity and the capacity for learning and memory formation. The silencing of genes encoding transcription factors involved in stress response, such as CREB (cAMP response element binding protein), would reduce the capacity of neural circuits to mount appropriate responses to stressful stimuli. (Liu et al., 2024) The combined effect of excessive ZNF124-

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

mediated transcriptional repression would be the creation of a neural environment characterized by reduced plasticity, impaired stress adaptation, and compromised behavioral flexibility. These neurobiological changes would predispose individuals to reliance on maladaptive coping mechanisms such as alcohol consumption, as normal adaptive responses would be functionally compromised.

The clinical relevance of this transcriptional dysregulation mechanism is supported by neurobiological research demonstrating that chronic alcohol exposure alters transcriptional programs in reward-related brain regions, including the prefrontal cortex and nucleus accumbens. Alcohol's acute effects involve disruption of glutamatergic and GABAergic neurotransmission, but chronic exposure produces adaptive changes in transcriptional regulation that constitute neuroadaptation (Hart & Kranzler, 2015). The identification of ZNF124-mediated transcriptional repression as a genetically-determined predisposing factor suggests that individuals carrying risk alleles at the ZNF124 locus may already possess baseline transcriptional profiles characterized by reduced expression of genes essential for adaptive neural function, even prior to alcohol exposure. This genetic predisposition may interact with alcohol's neuroadaptive effects to produce particularly severe alterations in transcriptional profiles and behavioral phenotypes in genetically susceptible individuals.

The Reactome pathway analysis identified protein deubiquitination as the most strongly enriched biological process, with USP12-mediated deubiquitination showing the highest odds ratios (36.95 to 52.40) among pathway-level findings. The biological significance of USP12-mediated dysregulation extends to multiple protein substrates critical for synaptic function and immune regulation. USP12 enzymatically removes ubiquitin conjugates from protein substrates, thereby regulating protein stability, localization, and functional activity. Dysregulation of USP12 deubiquitinating activity would produce imbalances in the steady-state levels of numerous substrates that accumulate or deplete depending on the direction of dysregulation.

Among the key substrates of USP12-mediated deubiquitination are the glutamate receptor subunits AMPA receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR). These ionotropic glutamate receptors are critical mediators of excitatory neurotransmission and activity-dependent synaptic plasticity. The ubiquitination status of AMPAR and NMDAR determines their trafficking to and from the synaptic membrane, their interaction with synaptic scaffolding proteins, and their functional association with intracellular signaling cascades. Dysregulated deubiquitination would impair the normal dynamic regulation of glutamate receptor trafficking, leading to altered synaptic strength and compromised mechanisms of long-term potentiation (LTP) and long-term depression (LTD) that underlie learning and memory formation. This would have direct consequences for the consolidation of learned associations between environmental cues and rewarding (or aversive) outcomes—a fundamental mechanism in the development of addiction.

A second major USP12 substrate is PSD-95 (postsynaptic density protein 95). This synaptic scaffolding protein organizes postsynaptic signaling complexes by simultaneously binding to glutamate receptors, voltage-gated ion channels, and intracellular signaling proteins. PSD-95 ubiquitination regulates its synaptic localization and functional capacity to organize postsynaptic signaling. USP12-mediated dysregulation of PSD-95 deubiquitination would alter the organisation and signalling capacity of postsynaptic densities, with downstream consequences for calcium signalling, kinase activation, and transcriptional responses to synaptic activity. These effects would further compromise the ability of neurons to implement activity-dependent synaptic modifications necessary for behavioral adaptation.

Beyond synaptic mechanisms, USP12 deubiquitination also regulates immune signalling proteins, including IFI16 (interferon-inducible protein 16), a pathogen recognition receptor involved in innate immune sensing, and NEMO (NF-kappa B essential modulator), a regulatory protein in the nuclear factor kappa B (NF-κB) signalling cascade. Dysregulated deubiquitination of these immune signaling proteins would impair the normal cascade of innate immune responses to pathogenic threats. This provides molecular explanation for the enrichment of HSV-1 pathway genes identified in the KEGG pathway analysis, as the same genetic variants affecting USP12 deubiquitinating activity would also dysregulate immune signaling cascades essential for antiviral responses.

The identification of HSV-1 infection pathway enrichment in the KEGG pathway analysis represents an unexpected but biologically meaningful finding that bridges alcohol dependence genetics to immunological dysfunction. While alcohol dependence is primarily conceptualized as a neuropsychiatric disorder, the enrichment of genes involved in antiviral immunity provides molecular evidence linking genetic predisposition to alcohol dependence with dysregulation

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

of innate immune responses. This finding aligns with epidemiological evidence demonstrating that individuals with alcohol dependence show increased susceptibility to herpes simplex virus infections, prolonged duration of active infections, and exaggerated inflammatory responses to viral reactivation (World Health Organization, 2018).

The mechanistic basis for the HSV-1 pathway enrichment involves the role of ZNF124 in transcriptional regulation of interferon-stimulated genes (ISGs) and upstream components of the toll-like receptor (TLR) signaling pathway. ZNF124-mediated transcriptional repression of genes encoding interferon-α and interferon-β would reduce the capacity of infected cells and immune cells to produce these critical antiviral cytokines. The repression of genes encoding downstream interferon-stimulated genes, including myxovirus resistance protein A (MxA), myxovirus resistance protein B (MxB), oligoadenylate synthase (OAS), and protein kinase R (PKR), would further impair antiviral protein synthesis and viral replication inhibition. The combined effect of impaired interferon production and reduced expression of antiviral effector proteins would create a state of severely compromised antiviral immunity.

The clinical consequences of this immune dysregulation are particularly relevant in the context of alcohol's known immunosuppressive effects. Chronic alcohol consumption independently suppresses innate immune responses through multiple mechanisms including reduction of toll-like receptor expression, impaired cytokine production, and altered natural killer cell function (World Health Organization, 2018). The convergence of genetic predisposition (ZNF124-mediated repression of immune genes) with environmental exposure (chronic alcohol consumption) produces a dual mechanism of immune dysregulation that exceeds the effects of either factor alone. This explains the clinical observation that individuals with alcohol dependence experience dramatically elevated infection rates and prolonged infection durations compared to the general population.

The microRNA enrichment analysis identified 35 microRNAs (9 experimentally validated, 7 computationally predicted, plus murine orthologs) that show enrichment for targeting the two hub genes ZNF124 and USP12. The exceptionally high odds ratios for validated microRNA targets (ranging from 84.96 to 768.62) indicate extremely high specificity of these regulatory relationships. The concentration of multiple microRNAs on the same target genes creates a regulatory architecture with biological redundancy—multiple independent regulatory mechanisms controlling the same gene products. This redundancy serves biological functions including buffering against genetic variation and fine-tuning of gene expression in response to developmental stage, tissue type, and environmental context.

The presence of dual-target microRNAs that simultaneously regulate both ZNF124 and USP12 represents a particularly elegant regulatory solution that ensures coordinated dysregulation of the two hub genes. If dysregulation of ZNF124 and USP12 occurred independently, the consequences for neural function would be unpredictable and potentially less severe. However, the presence of microRNAs targeting both genes simultaneously ensures that genetic variants affecting microRNA expression would produce coordinated changes in both transcriptional repression (through ZNF124) and post-translational dysregulation (through USP12). This coordinated dysregulation at two different biological levels would create a pathophysiological state with more severe consequences than either mechanism alone.

The evolutionary conservation of microRNA targeting across mammalian species, as evidenced by the presence of murine orthologs of several enriched microRNAs, indicates that these regulatory relationships represent functionally important and evolutionarily stable mechanisms. The conservation suggests that similar regulatory architectures operate in other mammalian species including laboratory rodents, enabling direct experimental validation of these microRNA-mediated regulatory relationships through genetic manipulation studies.

The comprehensive integration of findings from six independent analytical approaches yields a coherent model of alcohol dependence pathophysiology organized at multiple biological levels. At the genetic level, individual SNPs show modest effect sizes (odds ratios 1.15 to 1.22), consistent with the polygenic architecture of complex psychiatric traits. These modest individual genetic effects are progressively amplified through biological organization levels, with molecular function-level odds ratios reaching 40.82 to 52.40 for the hub genes, and post-transcriptional miRNA-level odds ratios reaching as high as 768.62. This progressive amplification of genetic signals through regulatory hierarchies demonstrates the importance of systems-level analysis for translating GWAS findings into mechanistic understanding.

The four identified biological mechanisms—transcriptional dysregulation, post-translational

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

modification dysregulation, post-transcriptional microRNA regulation, and immune dysregulation—operate synergistically rather than independently. The transcriptional repression mediated by ZNF124 affects the expression levels of genes encoding proteins subject to USP12-mediated deubiquitination, creating a coordinated alteration in both gene expression and protein stability. The post-transcriptional regulation by microRNAs controls the expression of both ZNF124 and USP12 themselves, providing feedback regulation that either amplifies or dampens the effects of genetic variants at the hub gene loci. The immune dysregulation mediated by both transcriptional repression of interferon genes and post-translational dysregulation of immune signaling proteins converges to impair antiviral defenses.

This multi-level integration produces a cascade from genetic variants through molecular mechanisms to clinical phenotypes. Individuals carrying risk alleles at the ZNF124 and USP12 loci would display baseline alterations in transcriptional programs (reduced expression of stress-response and plasticity genes), baseline alterations in protein stability (dysregulated levels of synaptic and immune proteins), and baseline alterations in post-transcriptional regulation (dysregulation of microRNA-mediated control systems). These baseline alterations would predispose to behavioral phenotypes including reduced behavioral flexibility, impaired stress coping, increased reward sensitivity to alcohol and impaired behavioral inhibition—all of which would promote progression toward problematic alcohol use patterns and alcohol dependence.

The findings from this functional enrichment analysis provide important context and novel mechanistic detail to previous genome-wide association studies of alcohol dependence and related phenotypes. Previous GWAS studies have identified multiple genetic loci associated with alcohol use disorder status, alcohol consumption quantity, and alcohol-related harm, with varying degrees of replication across ancestral populations (Mbarek et al., 2015; Sanchez-Roige et al., 2017; Sanchez-Roige et al., 2019; Walters et al., 2018). The twin and adoption study literature has consistently demonstrated substantial heritability of alcohol use disorders and problematic alcohol use, with estimates ranging from 50 to 60 percent in European ancestry populations (Verhulst et al., 2015). Behavioral genetic analyses using community samples have demonstrated genetic overlap between alcohol use and broader behavioral disinhibition phenotypes, including impulse control problems and substance use (Vrieze et al., 2013).

Previous functional studies have identified alcohol metabolizing genes, particularly aldehyde dehydrogenase 2 (ALDH2), as major genetic determinants of alcohol consumption in East Asian populations (Takeuchi et al., 2011; Quillen et al., 2013). The strong protective effects of ALDH2 loss-of-function variants in these populations reflect the enzyme's critical role in alcohol metabolism—loss of function increases acetaldehyde accumulation, producing aversive physiological effects that reduce alcohol consumption. However, ALDH2 variants have been documented in European ancestry populations as well, and European ancestry studies have identified additional loci including AUTS2 (autism susceptibility candidate 2), KLB (beta-Klotho), and multiple regulatory variants affecting alcohol consumption and dependence risk (Schumann et al., 2011; Schumann et al., 2016).

The current study advances beyond the identification of specific loci to provide mechanistic understanding through pathway-level analysis. Whereas previous GWAS identified statistically significant genetic variants and associated genes, functional enrichment analysis reveals which biological processes and molecular functions are disproportionately affected by the identified genetic variants. The concentration of enrichment on transcriptional repression and protein deubiquitination provides specificity about which cellular processes are dysregulated, enabling more targeted investigation of the neurobiological substrates underlying genetic predisposition.

The identification of specific molecular mechanisms underlying genetic predisposition to alcohol dependence has implications for understanding the heterogeneity of clinical presentation and treatment response. Alcohol dependence is clinically heterogeneous, with individuals showing varying patterns of drinking behavior, age of onset, severity of consequences, and response to different treatment modalities (American Psychiatric Association, 2013). The characterization of transcriptional dysregulation, post-translational dysregulation, post-transcriptional dysregulation, and immune dysregulation as distinct but integrated mechanisms suggests that different individuals may show different combinations of these dysregulation patterns depending on the specific genetic variants they carry.

Individuals with primary dysregulation of ZNF124-mediated transcriptional repression might show particular vulnerability to stress-induced drinking and difficulty with stress coping, as the transcriptional mechanisms necessary for stress adaptation would be compromised. Individuals with primary dysregulation of USP12-mediated protein deubiquitination might show particular

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

vulnerability to reward-seeking behaviors and difficulty with behavioral inhibition, as the synaptic mechanisms underlying learning and inhibitory control would be compromised. Individuals with dysregulation of microRNA-mediated control systems might show greater difficulty with compensating for genetic variants through learned adaptations, as the normal buffering mechanisms would be impaired. Individuals with immune dysregulation through compromised antiviral responses might show particular susceptibility to infection-triggered relapses, as concurrent viral infections would create additional physiological and psychological stressors.

This mechanistic understanding could enable more targeted treatment selection based on individual genetic profiles. If an individual is identified as carrying risk variants in ZNF124 with primary transcriptional dysregulation, treatment approaches emphasising stress management and the development of alternative coping mechanisms might be particularly beneficial. If an individual shows USP12-mediated dysregulation affecting synaptic plasticity, cognitive-behavioural therapy focused on habit extinction and behavioural retraining might be particularly effective. If an individual shows post-transcriptional dysregulation through altered microRNA expression, pharmacological approaches targeting microRNA biogenesis or function might be beneficial. If an individual shows immune dysregulation, management of concurrent infections and immune system support might be important adjuncts to primary addiction treatment.

The World Health Organisation and American Psychiatric Association have developed standardised diagnostic criteria and screening instruments for the assessment of alcohol use disorders. The AUDIT (Alcohol Use Disorders Identification Test) represents a validated instrument for systematic detection of harmful alcohol consumption and identification of individuals requiring intervention (Saunders et al., 1993; Babor et al., 2001). Previous GWAS studies have used AUDIT scores as quantitative phenotypes, identifying genetic variants associated with dimensional measures of problematic alcohol use (Mbarek et al., 2015; Sanchez-Roige et al., 2017; Sanchez-Roige et al., 2019). The current study's identification of molecular mechanisms affecting neurobiological substrates of reward, stress response, and behavioral control provides explanatory frameworks for understanding how genetic variants contribute to the elevated AUDIT scores and diagnostic criteria for alcohol use disorder.

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) defines alcohol use disorder through criteria including loss of control over drinking, continued drinking despite adverse consequences, tolerance and withdrawal symptoms, and neglect of other activities in favor of alcohol use (American Psychiatric Association, 2013). The previous DSM-IV-TR classification distinguished between alcohol abuse and dependence as separate diagnoses (American Psychiatric Association, 2000), while DSM-5 consolidates these into a single dimensional diagnosis of alcohol use disorder with severity specifiers. The molecular mechanisms identified in the current study—transcriptional dysregulation affecting behavioral control and stress adaptation, post-translational dysregulation affecting learning and habit formation, post-transcriptional dysregulation affecting regulatory buffering, and immune dysregulation affecting physiological resilience—contribute to multiple diagnostic criteria. These molecular mechanisms represent biological underpinnings of the clinical manifestations captured by diagnostic criteria.

The identification of specific genes and molecular pathways dysregulated in alcohol dependence enables systematic exploration of therapeutic targets. ZNF124, as a DNA-binding transcriptional repressor, represents a potential drug target for inhibition. Small molecule inhibitors that block ZNF124's DNA-binding capacity or disrupt its recruitment of corepressor complexes could reduce the excessive transcriptional repression, allowing restoration of expression for genes critical to stress adaptation and behavioral control. This approach would follow similar strategies used for targeting other disease-associated transcriptional regulators in oncology and other fields.

USP12, as a deubiquitinating enzyme, represents a potential target for substrate-selective inhibition. Rather than globally inhibiting all USP12 deubiquitinating activity (which would likely produce severe side effects), substrate-selective inhibitors could specifically block deubiquitination of particular protein substrates such as AMPA receptors or immune signaling proteins while leaving other substrates unaffected. This approach has precedent in the development of selective proteasome inhibitors that target the degradation of specific protein classes.

MicroRNA therapeutics represent another therapeutic approach targeting post-transcriptional dysregulation. AntagomiRs (chemically modified oligonucleotides that inhibit specific microRNAs) could suppress dysregulated microRNAs identified in the current study. Conversely, microRNA mimics could restore the expression of microRNAs whose levels are reduced in alcohol dependence. These approaches have shown promise in

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

clinical trials for various diseases and represent an emerging therapeutic modality applicable to alcohol dependence.

Immune modulation represents a fourth therapeutic axis. Anti-inflammatory agents, toll-like receptor antagonists, or interferon-enhancing strategies could improve dysregulated immune function and reduce infection susceptibility in individuals with alcohol dependence. This approach would be particularly beneficial for individuals showing the immune dysregulation phenotype identified in the current study.

Limitations and Future Directions

While the current study provides comprehensive functional enrichment analysis integrated across multiple bioinformatics platforms, several limitations warrant acknowledgment. First, the analysis identified associations between genetic variants and biological pathways based on statistical enrichment, but does not provide direct functional validation through experimental manipulation. Future studies would benefit from CRISPR-based gene editing approaches in neuronal cultures or whole-organism genetic models to directly test whether modulation of ZNF124, USP12, or specific microRNAs produces predicted changes in neurobiological function and behavioral phenotypes relevant to alcohol use.

Second, the study examined European ancestry populations, as the GWAS data underlying the analysis were primarily from European ancestry individuals. Validation in diverse ancestral populations would be important to determine whether the identified mechanisms operate similarly across genetic backgrounds and whether the identified genes represent universal mechanisms or show population-specific variation in functional effects. Previous work has identified substantial variation in genetic architecture across ancestral groups for alcohol dependence phenotypes, suggesting that population-specific analyses may reveal additional mechanistically important variants (Yang et al., 2014; Gelernter et al., 2013; Gelernter et al., 2018).

Third, the current analysis examined static cross-sectional data regarding genetic variation and pathway enrichment, but did not address temporal dynamics of molecular changes during alcohol exposure or across the progression from initial use to problematic use. Future studies incorporating longitudinal assessments of gene expression, protein levels, and microRNA abundance throughout the progression of alcohol use would provide insight into how genetic predisposition dynamically interacts with alcohol exposure to produce progressive neurobiological changes.

Fourth, the study identified enrichment in HSV-1 pathway genes as reflecting immune dysregulation, but did not investigate whether actual herpes simplex virus infection status correlates with alcohol dependence severity or treatment outcome. Future studies examining HSV-1 seropositivity in large samples of individuals with alcohol dependence could test whether viral infection history modifies the clinical phenotype or treatment response in genetically susceptible individuals.

Fifth, the study did not address potential gene-environment interactions where genetic variants' phenotypic effects depend on environmental exposures. Investigation of interactions between ZNF124 and USP12 variants and environmental stressors, nutritional status, early-life adversity, or concurrent substance use could reveal how genetic predisposition is modified by environmental context.

Future Research Directions

The identification of ZNF124 and USP12 as hub genes and the characterization of four dysregulation mechanisms suggests several concrete future research directions. Functional characterization studies should examine how genetic variants at the identified loci affect ZNF124 and USP12 expression levels and activity. Electrophoretic mobility shift assays could test whether SNPs create or eliminate transcription factor binding sites affecting ZNF124 expression. Luciferase reporter assays could test whether SNPs affect regulatory element activity. Mass spectrometry-based proteomics could characterize how USP12 activity affects the abundance of known substrates in neuronal cell systems and brain tissue.

Animal model studies should test whether CRISPR-mediated knockout or knockdown of Zfp36 (the murine ortholog of ZNF124) or Usp12 produces altered alcohol consumption, altered stress responsivity, or altered cognitive phenotypes in mice. Electrophysiological recording from neurons in knockout animals could characterize how ZNF124 or USP12 deletion affects synaptic plasticity mechanisms. Two-photon imaging could assess effects on dendritic spine density and dynamics as markers of synaptic remodeling.

Neuroimaging studies in human subjects should test whether genetic risk variants affecting ZNF124 and USP12 correlate with alterations in brain structure or function, particularly in regions involved in reward processing (ventral striatum, ventromedial prefrontal cortex), stress response (amygdala, dorsolateral prefrontal cortex) and behavioral control (anterior cingulate cortex, ventrolateral prefrontal cortex). Resting-state functional

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

connectivity analysis could characterize network-level changes associated with genetic variants.

Clinical translation studies should test whether ZNF124 and USP12 expression levels in peripheral blood or cerebrospinal fluid correlate with alcohol dependence severity, age of onset, or response to treatment. Such biomarker studies could enable risk stratification and treatment personalization.

Conclusions and Significance

The functional enrichment analysis of alcohol dependence-associated genetic variants identifies two hub genes (ZNF124 and USP12) operating through four integrated biological mechanisms (transcriptional repression, protein deubiquitination, post-transcriptional microRNA regulation, and immune dysregulation) to dysregulate neurobiological substrates of reward processing, stress response, behavioral control and immune function. The convergence of multiple independent analytical approaches on these findings, combined with the high positive predictive values (0.78-0.95) and reasonable sensitivity-specificity profiles (sensitivity 75-94%, specificity 85-97%), provides confidence that identified enrichments represent genuine biological mechanisms rather than statistical artefacts. The identification of specific dysregulation mechanisms enables more targeted investigation of pathophysiology, more rational development of therapeutics and more informed clinical prediction of treatment response.

The current findings extend the field beyond simple identification of disease-associated loci to provide mechanistic understanding of how genetic predisposition operates at molecular, cellular, and systems levels. This mechanistic understanding aligns with and extends upon the substantial literature on alcohol's neurobiological effects and the documented role of transcriptional and synaptic dysregulation in addiction across multiple drug classes. The proposed mechanisms are consistent with diagnostic criteria for alcohol use disorder as defined in the DSM-5 and the dimensional assessment approaches used in studies employing AUDIT scores and related quantitative measures of problematic alcohol use.

The work presented here represents important progress toward understanding the genetic basis of alcohol dependence and identifying actionable therapeutic targets. Continued investigation of ZNF124, USP12, and the 35 identified microRNAs through functional genetics, molecular biology, animal modeling, neuroimaging, and clinical research should yield insights into pathogenic mechanisms and opportunities for therapeutic intervention that could ultimately improve treatment outcomes for the millions of individuals worldwide affected by alcohol use disorder.

Declarations

Ethics Approval and Consent to Participate

This study is a secondary analysis of previously published and publicly available genome-wide association study (GWAS) summary statistics. All original studies included in the analysis obtained ethical approval from their respective institutional review boards and complied with the Declaration of Helsinki. Informed consent was obtained from all participants in the original studies. The present analysis did not involve direct contact with human participants and did not require additional ethical approval.

Availability of Data and Materials

All genome-wide association summary statistics analyzed in this study are publicly available from the original publications and the GWAS Catalog. Data sources are cited within the manuscript. All analytical methods and parameters are described in sufficient detail to ensure reproducibility.

Competing Interests

The authors declare that they have no competing interests.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Acknowledgements

The authors acknowledge the investigators and participants of the original genome-wide association studies whose publicly available data made this research possible. We also acknowledge the developers and curators of the Gene Ontology, KEGG, Reactome, miRTarBase, and TargetScan databases for providing essential resources for functional annotation and enrichment analysis.

References

American Psychiatric Association. (2000). *Diagnostic and statistical manual of mental disorders: DSM-IV-TR*. Author.

American Psychiatric Association. (2013). Diagnostic and Statistical Manual of Mental Disorders. https://doi.org/10.1176/appi.books.9780890425596

Babor, T. F., Higgins-Biddle, J. C., Saunders, J. B., & Monteiro, M. G. (2001). *AUDIT: The Alcohol*

Use Disorders Identification Test: Guidelines for use in primary health care (2nd ed.). World Health Organization.

Bierut, L. J. (2010). A genome-wide association study of alcohol dependence. *Proceedings of the National Academy of Sciences of the United States of America, 107*(11), 5082–5087. https://doi.org/10.1073/pnas.0911109107

Chaudhary, A.K., Sudhindra, P.A., & Rai, R.K. (2024). Advancements in methanol poisoning management: Integrating traditional treatments, modern innovations, and artificial intelligence for optimal patient outcomes. *Ro. J. Pharm. Pract*., *17*(1-2), 10-8. https://doi.org/10.37897/RJPhP.2024.1-2.2

Du, A., Chen, Y., Qiao, S., Dong, J., Li, Y., Cao, B., Zhao, R., & Zhang, R. (2024). Analysis of microRNAs and the microRNA-messengerRNA regulatory network in chronic alcohol exposure. *Front. Pharmacol., 15*, 1377501. https://doi.org/10.3389/fphar.2024.1377501

Gelernter, J., Kranzler, H. R., Sherva, R., Almasy, L., Koesterer, R., Smith, A. H., Anton, R., Preuss, U. W., Ridinger, M., Rujescu, D., Wodarz, N., Zill, P., Zhao, H., & Farrer, L. A. (2013). Genome-wide association study of alcohol dependence:significant findings in African- and European-Americans including novel risk loci. *Molecular Psychiatry, 19*(1), 41–49. https://doi.org/10.1038/mp.2013.145

Gelernter, J., Zhou, H., Nuñez, Y. Z., Mutirangura, A., Malison, R. T., & Kalayasiri, R. (2018). Genomewide Association Study of Alcohol Dependence and Related Traits in a Thai Population. *Alcoholism: Clinical and Experimental Research, 42*(5), 861–868. Portico. https://doi.org/10.1111/acer.13614

Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. J., … Zhang, J. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology, 5*(10). https://doi.org/10.1186/gb-2004-5-10-r80

Hart, A. B., & Kranzler, H. R. (2015). Alcohol dependence genetics: Lessons learned from genome-wide association studies (GWAS) and post-GWAS analyses. *Alcoholism: Clinical and Experimental Research, 39*, 1312–1327. https://doi.org/10.1111/acer.12792

Kapoor, M., Wang, J.C., Farris, S. P., Liu, Y., McClintick, J., Gupta, I., Meyers, J. L., Bertelsen, S., Chao, M., Nurnberger, J., Tischfield, J., Harari, O., Zeran, L., Hesselbrock, V., Bauer, L., Raj, T., Porjesz, B., Agrawal, A., Foroud, T., … Goate, A. (2019). Analysis of whole genome-transcriptomic organization in brain to identify genes associated with alcoholism. *Translational Psychiatry, 9*(1). https://doi.org/10.1038/s41398-019-0384-y

Liu, X., Dong, L., Jiang, Z., Song, M., & Yan, P. (2024). Identifying the differentially expressed peripheral blood microRNAs in psychiatric disorders: a systematic review and meta-analysis. *Front Psychiatry, 15*, 1390366. https://doi.org/10.3389/fpsyt.2024.1390366.

Mbarek, H., Milaneschi, Y., Fedko, I. O., Hottenga, J., de Moor, M. H. M., Jansen, R., Gelernter, J., Sherva, R., Willemsen, G., Boomsma, D. I., Penninx, B. W., & Vink, J. M. (2015). The genetics of alcohol dependence: Twin and SNP-based heritability, and genome-wide association study based on AUDIT scores. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics, 168*(8), 739–748. https://doi.org/10.1002/ajmg.b.32379

Morris, J., Boness, C. L., & Hartwell, M. (2025). Key Terms and Concepts in Alcohol Use and Problems: A Critical Evaluation. *Substance Use: Research and Treatment, 19*. https://doi.org/10.1177/29768357241312555

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., … Sham, P. C. (2007). PLINK: A tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics, 81*(3), 559–575. https://doi.org/10.1086/519795

Qiu, H., Shen, L., Shen, Y., & Mao, Y. (2023). Identification of a miRNA-mRNA regulatory network for post-stroke depression: a machine-learning approach. *Front Neurol., 14*, 1096911. https://doi.org/10.3389/fneur.2023.1096911.

Quillen, E. E., Chen, X., Almasy, L., Yang, F., He, H., Li, X., Wang, X., Liu, T., Hao, W., Deng, H., Kranzler, H. R., & Gelernter, J. (2013). ALDH2 is associated to alcohol dependence and is the major genetic determinant of "daily maximum drinks" in a GWAS study of an isolated rural chinese sample. *American Journal of Medical*

Genetics Part B: Neuropsychiatric Genetics, 165(2), 103–110. https://doi.org/10.1002/ajmg.b.32213

Ramirez-Roman, M. E., Serrano-Rodríguez, A., & Bayés-Génés, A. (2018). Epigenetic mechanisms of alcohol neuroadaptation. *Substance Abuse: Research and Treatment, 12*, 1179069518779809. https://doi.org/10.1177/1179069518779809

Sanchez-Roige, S., Fontanillas, P., Elson, S. L., Gray, J. C., de Wit, H., Davis, L. K., MacKillop, J., & Palmer, A. A. (2017). Genome-wide association study of alcohol use disorder identification test (AUDIT) scores in 20 328 research participants of European ancestry. *Addiction Biology, 24*(1), 121–131. https://doi.org/10.1111/adb.12574

Sanchez-Roige, S., Palmer, A. A., Fontanillas, P., Elson, S. L., Adams, M. J., Howard, D. M., Edenberg, H. J., Davies, G., Crist, R. C., Deary, I. J., McIntosh, A. M., & Clarke, T.-K. (2019). Genome-Wide Association Study Meta-Analysis of the Alcohol Use Disorders Identification Test (AUDIT) in Two Population-Based Cohorts. *American Journal of Psychiatry, 176*(2), 107–118. https://doi.org/10.1176/appi.ajp.2018.18040369

Saunders, J. B., Aasland, O. G., Babor, T. F., de la Fuente, J. R., & Grant, M. (1993). Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO collaborative project on early detection of persons with harmful alcohol consumption—II. *Addiction, 88*, 791–804. https://doi.org/10.1111/j.1360-0443.1993.tb02093.x

Savarese, A. M., Lasek, A. W., & Reifman, A. (2018). Transcriptional regulators as targets for alcohol use disorder treatment. *Drug Discovery Today, 23*(11), 1912–1922.

Schumann, G., Coin, L. J., Lourdusamy, A., Charoen, P., Berger, K. H., Stacey, D., Desrivières, S., Aliev, F. A., Khan, A. A., Amin, N., Aulchenko, Y. S., Bakalkin, G., Bakker, S. J., Balkau, B., Beulens, J. W., Bilbao, A., de Boer, R. A., Beury, D., Bots, M. L., … Elliott, P. (2011). Genome-wide association and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. *Proceedings of the National Academy of Sciences, 108*(17), 7119–7124. https://doi.org/10.1073/pnas.1017288108

Schumann, G., Liu, C., O'Reilly, P., Gao, H., Song, P., Xu, B., Ruggeri, B., Amin, N., Jia, T., Preis, S., Segura Lepe, M., Akira, S., Barbieri, C., Baumeister, S., Cauchi, S., Clarke, T.-K., Enroth, S., Fischer, K., Hällfors, J., … Elliott, P. (2016). KLB is associated with alcohol drinking, and its gene product β-Klotho is necessary for FGF21 regulation of alcohol preference. *Proceedings of the National Academy of Sciences, 113*(50), 14372–14377. https://doi.org/10.1073/pnas.1611243113

Takeuchi, F., Isono, M., Nabika, T., Katsuya, T., Sugiyama, T., Yamaguchi, S., Kobayashi, S., Ogihara, T., Yamori, Y., Fujioka, A., & Kato, N. (2011). Confirmation of ALDH2 as a Major Locus of Drinking Behavior and of Its Variants Regulating Multiple Metabolic Phenotypes in a Japanese Population. *Circulation Journal, 75*(4), 911–918. https://doi.org/10.1253/circj.cj-10-0774

Verhulst, B., Neale, M. C., & Kendler, K. S. (2015). The heritability of alcohol use disorders: A meta-analysis of twin and adoption studies. *Psychological Medicine, 45*(5), 1061–1072. https://doi.org/10.1017/S0033291714002165

Vrieze, S. I., McGue, M., Miller, M. B., Hicks, B. M., & Iacono, W. G. (2013). Three mutually informative ways to understand the genetic relationships among behavioral disinhibition, alcohol use, drug use, nicotine use/dependence, and their co-occurrence: Twin biometry, GCTA, and genome-wide scoring. *Behavior Genetics, 43*, 97–107. https://doi.org/10.1007/s10519-013-9584-z

Walters, R. K., Polimanti, R., Johnson, E. C., McClintick, J. N., Adams, M. J., Adkins, A. E., Aliev, F., Bacanu, S.A., Batzler, A., Bertelsen, S., Biernacka, J. M., Bigdeli, T. B., Chen, L.-S., Clarke, T.K., Chou, Y.L., Degenhardt, F., Docherty, A. R., Edwards, A. C., … Agrawal, A. (2018). Transancestral GWAS of alcohol dependence reveals common genetic underpinnings with psychiatric disorders. *Nature Neuroscience, 21*(12), 1656–1669. https://doi.org/10.1038/s41593-018-0275-1

Willis, C., White, J. D., Minto, M. S., Quach, B. C., Han, S., Tao, R., Shin, J. H., Deep-Soboslay, A., Hyde, T. M., Mayfield, R. D., Webb, B. T., Johnson, E. O., Kleinman, J. E., Bierut, L. J., & Hancock, D. B. (2024). Gene expression differences associated with alcohol use disorder in human

brain. *Molecular Psychiatry, 30*(4), 1617–1626. https://doi.org/10.1038/s41380-024-02777-1

World Health Organization (2018). Global status report on alcohol and health 2018.

Yang, C., Li, C., Kranzler, H. R., Farrer, L. A., Zhao, H., & Gelernter, J. (2013). Exploring the genetic architecture of alcohol dependence in African-Americans via analysis of a genome wide set of common variants. *Human Genetics, 133*(5), 617–624. https://doi.org/10.1007/s00439-013-1399-8

Zhou, H., & Gelernter, J. (2024). Human genetics and epigenetics of alcohol use disorder. *J. Clin. Invest., 134*(16), e172885. https://doi.org/10.1172/JCI172885.

How to cite this Article:

Arbind Kumar Chaudhary, Anamitra Hait and Avinash S.S. (2025). Multi-Level Functional Enrichment Analysis Reveals Transcriptional Repression and Post-Translational Dysregulation as Central Mechanisms in Alcohol Dependence Genetic Architecture: A 640-Fold Signal Amplification Cascade from GWAS Loci to MicroRNA Regulation. *International Journal of Experimental Research and Review*, *49*, 19-48.

DOI: https://doi.org/10.52756/ijerr.2025.v49.003

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.